International Society for Biological Therapy of Cancer 24th Annual Meeting Abstracts

(Primary Authors are Italicized)

ADOPTIVE TRANSFER

Adoptive Transfer Immunotherapy of Cancer Patients According to Lymphocytes Phenotyping Plus Tumor Markers Monitoring

*Hannes Anthopoulos**, Konstantinos Papapolychroniadis†, Epaminontas Fahantidis†, Daniil Paramythiotis†, Parisis Makrantonakis‡. **K-Bio Clinical Biochemical Institute for Cell Biotechnology and Immunology, Munich, Germany; †First Surgical Propedeutic Clinic; ‡Department of Medical Oncology, First Medical Clinic, Aristotle University of Thessaloniki, Thessaloniki, Greece.*

Active immunologic therapy (AHICE) is a fully scientifically recognized, new therapeutic method among the optional adjuvant approaches to the management of cancer patients.

Basically, AHICE is a special kind of hemodialysis—after a special biochemical demasking of the tumor cells (eg, eliminating of the escape mechanisms of the tumor cells membrane. These escape mechanisms are the reason why cancer is establishing in the body despite the existence of a healthy immune system). Only after that, the immune system of the patients will be able to spontaneously detect, identify selectively the pathologically altered tumor cells, eliminate them through apoptosis as none histocompatible cell forms! This elimination of cancer cells is succeeded by a well-coordinated but very complex immune-biochemical mechanism!

That autologous immune response eliminate everywhere, the patient's body, the recognized none histocompatible cancer cells through his unique selectivity, detection-ability intelligence.

In this study, we report the results of complete lymphocytes phenotyping and tumor markers monitoring in relation to outcomes of AHICE immune therapy in 25 breast, pancreas, lung, prostate, kidney, penis, bladder, ovarian cancer patients. Each AHICE cycle therapy was of the long-term therapy type (90 days). Before starting, every 3 weeks during AHICE therapy white blood count, lymphocyte phenotype (T lymphocytes, B lymphocytes, helper T4 cells, suppressor T8 cells, T4/T8 index, natural killer cells, HLA-DR-lymphocytes), and also the related concentration of tumor markers relative to primary malignancy (CEA, CA19-9, CA15-3, CA125, PSA, etc.), were examined.

In conclusion, after the analysis of our blood research study results, we can infer that after a earlier demasking of the tumor cells, the so-activated immune-competent lymphocytes for active immune tumor therapy are the significant points of reference for successful therapy.

Eradication of Established CD19-positive Leukemia Using A Single Injection of Chimeric Immunoreceptor Modified Lentiviral-Transduced T Cells in a Xenograft NOG Mouse Model

*David M. Barrett**, Carmine Carpenito†, Yang Bing Zhao†, Michael Kalos†, Carl June†, Stephan Grupp*. **Division of Oncology, Children's Hospital of Philadelphia; †Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA.*

CD19 is a membrane glycoprotein found on human B lymphocytes at all stages of maturation, and is not lost during malignant transformation to precursor B-cell ALL. T cells engineered with chimeric immunoreceptors (CIRs) consisting of an extracellular scFv domain against CD19 fused to the T-cell receptor ζ signal transduction domain represent a promising adoptive immunotherapy against leukemias refractory to traditional chemotherapy. In studies before our group, other researchers have shown that the inclusion of signaling domains from costimulatory molecules, such as CD28 or CD137 (4-1BB), can improve the antileukemic efficacy and in vivo persistence of CIR-modified T cells. To critically evaluate the antitumor efficacy of anti-CD19–targeting CIRs that contained various signaling domains alone or in combination we used the highly immunodeficient NOG mouse model, an aggressive $CD19^+$ leukemia cell line (Nalm-6), then treated with primary human $CD8^+$, $CD4^+$ lymphocyte genes modified to express anti-$CD19^-$ targeting CIRs with TcRζ alone, CD28-TcRî, CD137-TcRζ, $CD28^-$ CD137-TcR signaling domains.

NOG mice were injected with 1×10^6 Nalm-6 on day 1, a dose that produces 100% engraftment in the bone marrow by day 7 (95% confidence interval: 0.15%–1.2% of total bone marrow cells $CD19^+$) with 100% mortality by day 25 if untreated. T cells were expanded ex vivo using CD3/CD28 beads, subjected to lentiviral gene transfer of various CIRs, 1×10^7 T cells were injected on day 7 (70% CIR +). Mice were followed weekly by quantitative flow cytometric analysis of peripheral blood for CD3, CD4, CD8, CD19-positive human cells.

Mice treated with T cells engineered to express each of the anti-CD19 CIRs showed statistically significant improved survival over control animals, with animals succumbing either to leukemia, or in a construct-dependent manner, to xenogenic GVHD. In addition, a hierarchy of survival advantage was observed among the anti-CD19 constructs, with the CD19-CD28-CD137-ζ CIR mediating the most enhanced survival, with 37% of those mice surviving until the end of the study at day 125.

These data indicate that it is possible to eradicate established bone marrow disease with a single injection of lentiviral-transduced CIR T cells. Future efforts will focus on evaluating a model in which mice that appear cured will be rechallenged with leukemia to evaluate in vivo persistence and the surveillance capabilities of these CIR T cells.

TCR Against Cancer Testis Antigen MAGE-A3 for Targeted Adoptive Immunotherapy of Cancer

*Nachimuthu Chinnasamy**, Jennifer A. Wargo*†, Zhiya Yu*, Timothy L. Frankel*, John P. Riley*, Maria R. Parkhurst*, Nicholas P. Restifo*, Paul F. Robbins*, Steven A. Rosenberg*, Richard A. Morgan*. **Surgery Branch, National Cancer Institute, Bethesda, MD; †Department of Surgery, Harvard Medical School, Boston, MA.*

Targeted adoptive immunotherapy against melanocyte differentiation antigens can mediate objective tumor regression in melanoma patients. However, it is also associated with on-target toxicity to normal tissue including skin, eye, ear (Johnson et al, Blood, May 18, 2009). Targeting cancer testis antigens may lessen on-target toxicity as their expression is limited to tumor and non-MHC–bearing germ cell tissues. Cancer testis antigen MAGE-A3 is a member of the Melanoma Antigen Gene (MAGE) super family, is expressed in over 70% of metastatic melanomas and a wide range of nonmelanoma epithelial malignancies. We used a transgenic mouse model that expresses the human class I HLA-A2 molecule to generate reactive T-cell clones against the human MAGE-A3 antigen. Mice were immunized with 2 HLA-A2–restricted epitopes of MAGE-A3: 112-120 (KVAELVHFL) or 271–279 (FLWGPRALV) peptide with a helper peptide (HBVc:

128–140). T-cell clones were generated in vitro from the splenocytes after repeated stimulation with peptides. TCR α, β chains were isolated, tested for reactivity, and cloned into a MSGV1-based retroviral vector. Expression of both TCRs in human peripheral blood lymphocytes showed antigen-specific reactivity against a range of melanoma and nonmelanoma tumor cell targets. On the basis of improved reactivity against target cells, the TCR against MAGE-A3: 112–120 was selected for further development. This TCR is functional only in CD8 cells, not in CD4 cells. Peptide epitopes from both MAGE A3, A12 were efficiently recognized by the TCR-engineered peripheral blood lymphocytes. On the basis of these results, we are proposing a clinical trial targeting MAGE-A3 against a broad spectrum of cancers.

Adoptive Cell Therapy Using T Cells Expressing a Chimeric Antigen Receptor for Vascular Endothelial Growth Factor Receptor-2 Promotes Tumor Destruction and Enhances Immunotherapy in Mice

*Dhanalakshmi Chinnasamy**, Zhiya Yu*, Marc R. Theoret*, Rajeev K. Shrimali*, Yang Bing Zhao†, Nicholas P. Restifo*, Steven A. Rosenberg*. **Surgery Branch, National Cancer Institute, Bethesda, MD; †Department of Pathology, Laboratory Medicine, Abramson Cancer Research Institute, School of Medicine, University of Pennsylvania, Philadelphia, PA.*

Vascular endothelial growth factor (VEGF) plays an important role in vascular permeability and tumor angiogenesis. VEGF transduces signals mainly through VEGFR-2, which is over-expressed on tumor endothelial cells. We hypothesized that an adoptive cell therapy strategy targeting VEGFR-2 on tumor endothelial cells could inhibit tumor growth and metastases. To test this hypothesis, we constructed a series of recombinant retroviral vectors encoding a chimeric antigen receptor (CAR) comprising single-chain variable regions of an antimouse VEGFR-2 antibody (DC101; Imclone Systems Inc) linked to intracellular mouse T-cell signaling sequences derived from CD28, 4-1BB molecules, and CD3ς chains of the T-cell receptor. DC101-CAR-expressing vectors, under optimal conditions efficiently transduced mouse T cells (~75% to 90%). The DC101-CAR expressing T cells exhibited antigen-specific proliferation, interferon-γ secretion in vitro in response to plate bound VEGFR-2 protein as well as VEGFR-2 expressing mouse endothelial tumor cells. When adoptively transferred into lymphodepleted C57BL/6 mice bearing preestablished tumors, the DC101-CAR engineered mouse T cells strongly inhibited the growth of B16 melanoma and MC38 colon cancers significantly prolonged the survival of tumor-bearing animals without any treatment-related adverse toxicities. The in vivo antitumor activity of DC101-CAR modified T cells directly correlated with number-infused T cells. The presence of 4-1BB intracellular signaling sequences did not improve the therapeutic effectiveness of the CAR in T cells, but had a positive effect on their long-term persistence in vivo at the tumor site. Furthermore, intracellular signaling sequences are important for the antitumor activity of DC101-CAR, as their deletion can lead to complete loss of function of CAR both in vitro and in vivo without any compromise to their cell surface expression in transduced T cells. The DC101-CAR-modified T cells showed increased tumor infiltration compared with the control vector-transduced T cells. Overall, this preclinical study showed that adoptive cell therapy using a CAR against VEGFR-2 can mediate tumor suppression in several mouse tumor models, thus providing the preclinical justification for applying this approach to the treatment of human cancer.

Adoptive Transfer of T Cells Genetically Modified Using the Sleeping Beauty System

Laurence J. Cooper. Pediatrics, M.D. Anderson Cancer Center, Houston, TX.

Clinical trials have shown some therapeutic successes after the infusion of T cells genetically modified to be tumor specific on the expression of a chimeric antigen receptor (CAR). The therapeutic potential of infused CAR+ T cells is tied to their in vivo persistence, ability to be fully activated upon binding to the tumor-associated cell-surface antigen, independent of major histocompatibility

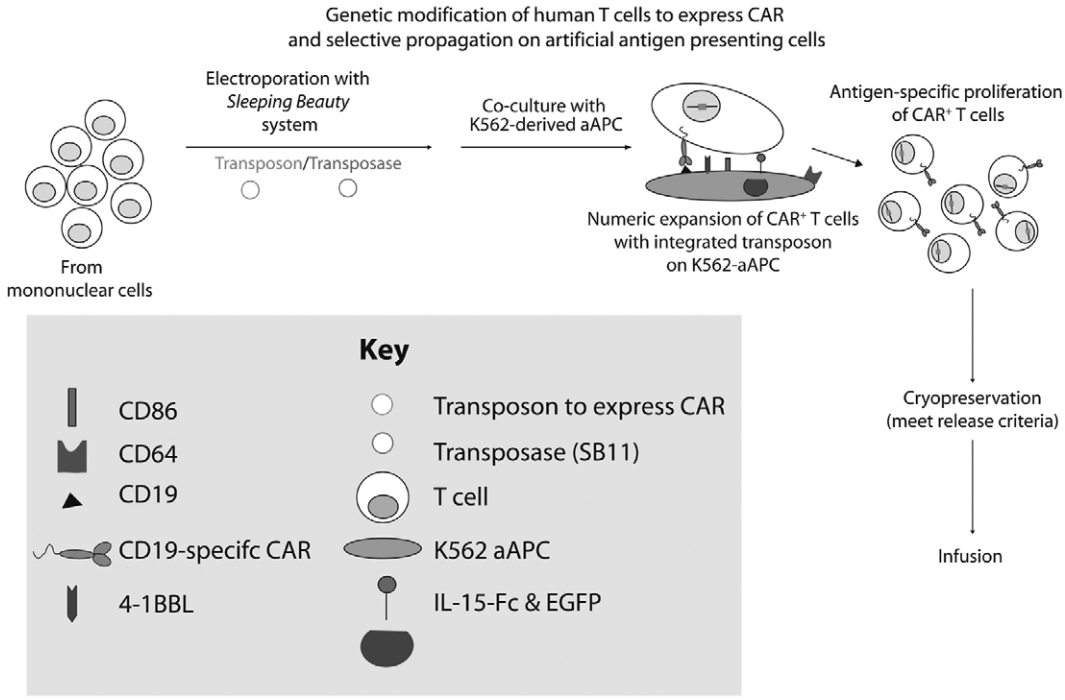

FIGURE 1 (Cooper). Schematic diagram showing nonviral gene transfer of the SB system genetically modified T cells.

complex, and within the malignant microenvironment. Thus, not only must gene transfer introduce a desired immunoreceptor, but T cells should be manipulated ex vivo to maintain their replicative potential and effector functions. To fulfill the promise of T cells as biologic therapies, iterative changes are needed to genetically modify, then remodify T cells as we progress from the bench to the bedside, and back again. We have used the Sleeping Beauty (SB) transposon/transposase system as a facile approach to genetically modifying T cells, derived from peripheral, neonatal blood, to introduce 1 or more CARs as well as other immunomodulatory imaging transgenes. When SB transposition of T cells is combined with their selected propagation on artificial antigen-presenting cells (aAPC), generated from K562 (in collaboration with Dr Carl June), we can reliably produce CAR+ T cells that have desired specificity, that show sustained proliferation and, can function as effector cells within tumor microenvironments. This technology can be translated into clinical practice using both off-the-shelf DNA plasmids; aAPC as defined by the standard operating procedures, which makes this methodology straightforward and cost-effective. We have adapted the SB system for its first-in-human use to generate a master cell bank of aAPC (by Production Assistance for Cellular Therapies under the auspices of National Heart, Lung and Blood Institute) manufacturing clinical-grade SB DNA plasmids. These platform technologies should enable us to infuse clinical-grade CAR+ T cells, such as with the specificity for CD19 on malignant B cells (Fig. 1). Hopefully, this approach to gene transfer and propagation will enable us and other researchers to undertake a series of human clinical trials infusing genetically modified T cells as well as other lymphocyte populations to improve the efficacy of adoptive immunotherapy. The plasticity of our approach based on the nonviral gene transfer of SB plasmids may be important not only for gene therapy trial development in the United States, but can be considered as an opportunity for other countries to genetically manipulate human cells in compliance with current good manufacturing practices.

[See Figure 1, Previous page.]

Immune Responses to Murine T-cell Receptors in Patients Enrolled in the TCR Gene Therapy Trials

Jeremy L. Davis, Marc Theoret, Zhili Zheng, Steven A. Rosenberg, Richard A. Morgan. *Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.*

Immune responses to gene-modified T lymphocytes are an established phenomenon in the field of human gene therapy in which host recognition and subsequent destruction of engineered cells can be a clinically significant impediment to effective therapy. We conducted 2 clinical trials in which cancer patients were treated with autologous lymphocytes genetically engineered to express murine T-cell receptors (mTCRs) specific for human tumor-associated antigens p53 and gp100 (154). Patients treated with a lymphodepleting chemotherapy regimen followed by gene-modified cell infusion, high-dose interleukin-2 experienced objective tumor response rates of 9% (1 of 11) and 19% (3 of 16), respectively. In 3 of 26 (12%) patients, posttreatment sera mediated a 60% to 99% inhibition of mTCR activity as measured by a reduction in antigen-specific IFN-γ release in coculture assays. In addition, in 6 (23%) patients posttreatment sera exhibited specific binding of human anti-mTCR antibodies to lymphocytes transduced with the mTCRs with which they were treated. On the basis of a lack of cross-reactivity with other mTCRs, the anti-mTCR antibodies did not seem to recognize the constant region. In 1 patient treated with the p53 TCR, posttreatment serum antibody also bound a different mTCR with a homologous β chain variable region, implicating this as the antibody-binding domain. In a parallel clinical trial using a fully human TCR, a humoral immune response to autologous gene-engineered cells was not observed in any of the 17 patients tested. Overall, the development of a host immune response was not correlated with the level of transduced cell persistence or response to therapy. In summary, patients treated with a mTCR can develop an immune response to gene-modified cells in a minority of cases. The impact of this occurrence in the setting of a modestly successful gene therapy program has yet to be determined.

Role of Complement in T-cell Tumor Infiltration and Rejection

*Andrea Facciabene**, John Facciponte*, Klara Balint*, Ronald Buckanovich†, Robert Deangelis‡, Paola Magotti‡, John Lambris‡, George Coukos*. **Ovarian Cancer Research Center; ‡Department of Pathology, University of Pennsylvania, Philadelphia, PA; †Medicine, University of Michigan, Ann Arbor, MI.*

The central role of the endotheliumin the complex scheme of the immunoresponse is becoming clear. We microdissected the endothelium of tumors with T-cell infiltrate (TIL) and tumors without TIL and performed a microarray analysis to define the pattern of protein expression of the 2 specimens. Within the different expressed proteins, we observed a striking increase of complement C3 in the endothelium of tumors with TIL. To investigate the role of C3 in TIL and its functions in tumor, we performed a lethal challenge using human papillomavirus E6/7 TC1 cells in C57 black 6 background C3KO, littermates. Around 5 to 8 days later, $CD3^+$ positive T cells from E7-immunized Wild Type (WT) C57 black 6 mice were adoptively transferred into the C3KO TC1 tumor-bearing mice. The absence of C3 expression from the host results in an almost complete abrogation of the E7 tetramer-positive TIL and in the loss of the tumor delay growth. Next, we used mice deficient for decay accelerating factor (DAF) or WT mice, repeated the initial experiment, performing the TC1 tumor challenge, the T cells adoptive transfer as explained for the C3KO model. To compare the efficacy of T-cell therapy in DAFKO and WT, 2 doses of T cells (3×10^6 or 1×10^7) were transferred from WT immunized mice in 2 different cohorts of tumor-bearing DAFKO mice. As expected, we observed the effects on tumor growth at the lowest dose of T-cell transfer (3×10^6 $CD3^+$) only in the DAFKO mice but not in the WT mice. These results confirm the central role of the complement activation in TIL into the tumor. The complement activation products C3a, C5a, known as the anaphylatoxins are potent proinflammatory chemoattractants. We sought to understand whether C5a were directly involved in T-cell tumor infiltration and rejection. We used 2 different approaches using T-cell adoptive transfer or vaccination in combination with the administration of a C5a antagonist (C5aRA). The administration of C5aRA drastically decreased the effects of both the treatments. To investigate the effects of C3 up-regulation in endothelial cells and T-cell interaction, we used an in vitro adhesion assay. Human umbilical vein endothelial cells (HUVEC) were seeded on a 96-well plate, treated overnight with C3a, C5a alone or in combination with 10-time excess of the specific antagonist. Treatment of HUVEC with C5a or C3a increases T-cell adhesion, the adhesion increase was abrogated by the specific antagonist. This work outlines the unknown role played by the complement in tumor TIL and endothelium activation.

Evidence for CD4 T-cell – mediated Tumor Regression, the Mechanism of the Subsequent Tumor Immune Escape

Kevin M. Friedman, James C. Yang, Peter Prieto, Laura E. Devillier, Steven A. Rosenberg, Mark E. Dudley. *Surgery Branch, National Cancer Institute, Bethesda, MD.*

Tumor-specific T cells can be obtained from tumor-infiltrating lymphocytes (TILs), which are rapidly expanded to large numbers, adoptively transferred into patients bearing advanced melanoma. After nonmyeloablative lymphopenic conditioning and treatment with a TIL cell product, more than 50% of the patients experienced an objective clinical response. Current research is focused on strategies that simplify the treatment and augment antitumor efficacy. Minimally cultured TILs are generally less differentiated, contain a higher frequency of CD4 T cells and then TIL cultures

are maintained for longer times in vitro. However, the contribution of CD4 T cells to the antitumor response is poorly understood. The immunologic, clinical findings from a patient who was treated with 2 courses of TIL cell products after lymphopenic conditioning are discussed here. The patient first received a minimally cultured T-cell product with antitumor activity that was completely blocked by a pan − HLA-DR blocking antibody. She experienced an objective clinical response highlighted by a dramatic reduction in her liver and splenic metastatic disease. The response was transient due to progression at pelvic sites and the development of an ovarian metastasis, which was subsequently resected. Although the treatment with T cells failed to recognize this recurrent ovarian tumor, antitumor activity could be reestablished with the ectopic expression of HLA-DR, mediated by ectopic transduction with the gene encoding the class II transactivator. The addition of a pan − HLA-DR blocking antibody, but not a pan-HLA class I antibody, completely inhibited the in vitro antitumor function. These data strongly suggest that tumor regression was mediated by the infused CD4 T cells and that the recurrent tumor escaped destruction by eluding CD4 recognition. A second TIL product derived from the recurrent tumor, consisting entirely of tumor-specific CD8 T cells was then administered. The patient experienced another objective response, which is ongoing at the time of this publication. Taken together, this report highlights the potential contribution of tumor-specific CD4 T cells in TILs for successful immunotherapy, informs future clinical trials.

Improving Tumor Infiltrating Lymphocyte Survival, Function for Melanoma Adoptive T-cell Therapy Through 4-1BB Costimulation

*Jessica A. Hernandez**†, Yufeng Li*†, Yijun Wang*, Patrick Hwu*†, Laszlo Radvanyi*†‡. **Melanoma Medical Oncology; ‡Breast Medical Oncology, University of Texas M.D. Anderson Cancer Center; † University of Texas Graduate School of Biomedical Sciences, Houston, TX.*

Adoptive T-cell therapy (ACT) using expanded tumor-infiltrating lymphocytes (TILs) with high-dose interleukin-2 (IL-2) is a promising form of tumor immunotherapy for stage IV melanoma showing clinical response rates of 50% or more in clinical trials. ACT requires the expansion of TILs ex vivo over a number of weeks culminating in a large-scale "rapid expansion protocol" (REP) using anti-CD3 antibody, peripheral blood mononuclear cell feeder cells, and IL-2 generating the TIL infusion product consisting of billions of antitumor $CD8^+$ T cells. During ex vivo expansion, $CD8^+$ TILs increase in number, differentiate, acquire an antitumor killing function, a process critical for tumor control after TIL infusion. We found many tumor-specific $CD8^+$ T cells lose the costimulatory molecule CD28 and become sensitive to "activation-induced cell death" (AICD) during T-cell receptor (TCR) stimulation. As CD28 is a costimulatory molecule preventing AICD in T cells, we sought to determine whether members of the tumor necrosis factor-R (TNF-R) family on expanded TIL could serve as alternative costimulatory molecules preventing AICD and sustain effector function. 4-1BB is a TNF-R family member active in peripheral blood $CD8^+$ T cells, but has never been studied in TILs. Here, we sought to determine the expression of 4-1BB on post-REP $CD8^+$ TILs, whether 4-1BB could be an alternative costimulatory pathway in $CD28^-$ TILs preventing AICD and sustaining an antitumor effector function. During the REP, we found that many $CD8^+$ TILs induced 4-1BB expression as CD28 expression was lost. When post-REP melanoma TILs were cultured in IL-2, restimulated through the TCR using anti-CD3, 4-1BB was further upregulated. We tested whether 4-1BB ligation could prevent AICD, facilitate post-REP TIL expansion, effector function using a commercially available agonistic anti − 4-1BB monoclonal antibody, a humanized anti − 4-1BB currently being tested in clinical trials. In both cases, ligation of 4-1BB during TCR restimulation with anti-CD3 on CD8+ TILs inhibited AICD, as measured by decreased Annexin V and 7-AAD staining. 4-1BB coligation in post-REP TILs facilitated the expansion of post-REP TILs over a 7-day period, improved anti-tumor CTL activity. This was associated with increased antiapoptotic gene (bcl-2, bcl-xL) expression, increased granzyme B, and perforin expression. Our results indicate that 4-1BB costimulation may improve TIL survival during melanoma ACT and boost antitumor cytolytic activity. This hypothesis will be tested in an upcoming phase I ACT clinical trial using humanized anti − 4-1BB antibody therapy after TIL and IL-2 infusion.

Mechanisms of CD8 Memory Loss to Neuroblastoma After Autologous Hematopoietic Stem Cell Transplantation

*Weiqing Jing**†, Bryon Johnson*†. **Pediatrics; †Cancer Center, Medical college of Wisconsin, Milwaukee, WI.*

We have shown that a multifaceted immunotherapeutic approach involving hematopoietic stem cell transplantation (HSCT), T-cell adoptive transfer, and cell-based tumor vaccination eliminates established neuroblastomas in murine models. One important observation was that the depletion of $CD4^+$ T cells correlated with increased CD8-mediated antitumor reactivity after HSCT if the transferred T cells had been presensitized to tumor antigens, but long-term tumor immunity failed to develop in these animals. To investigate the mechanism of CD8 memory loss in CD4-depleted hosts, a phenotypic comparison of $CD8^+$ T cells in CD4-depleted, control HSCT hosts was performed. We observed that PD-1 expression, a marker associated with T-cell exhaustion, was significantly upregulated on $CD8^+$ T cells in HSCT host mice with depleted $CD4^+$ T cells compared with control HSCT mice. Furthermore, $CD8^+$ T cells from CD4-depleted mice coexpressed multiple inhibitory receptors such as Lag3, Tim3, and 2B4 though not CTLA-4. PD-1^+ $CD8^+$ T cells were enriched with Annexin-V+ cells and had lower expression of interleukin-7 and interleukin-15 receptors; Bcl-2 had increased caspase-3 activation indicative of peripheral T-cell apoptosis. However, PD-1^+ $CD8^+$ T cells collected 21 days after transfer (peak PD-1 expression) had greater interferon-γ production and proliferative capacity than PD-1^- $CD8^+$ T cells contrasting characteristically exhausted PD-1^+ T cells generated via chronic antigen stimulation. Blockade of the PD-1/PD-L1 inhibitory pathway using anti − PD-L1 in CD4-depleted mice failed to restore CD8 antitumor memory. Anti − PD-L1 blockade further induced the expression of inhibitory receptors, the proliferative marker Ki-67, as well as increased apoptosis of $CD8^+$ T cells. Our results suggested that $CD8^+$ T cells in CD4-depleted host mice that proliferated rapidly and spontaneously, temporarily maintained function but ultimately generated an "exhausted" phenotype, underwent apoptosis, and resulted in $CD8^+$ T-cell memory loss. Defining the pathway involved in $CD8^+$ T-cell apoptosis after transfer to CD4-depleted hosts after HSCT will help us understand the mechanism of memory loss and aid in the exploration of new strategies to restore memory.

Epstein-Barr Virus-specific T Cells as Therapy for Relapsed/Refractory EBV-positive Lymphomas

*Kimberley Jones**, Frank Vari*, Rajiv Khanna†, Erica Han*, Sanjleena Singh*, David Ritchie‡, Maher K. Gandhi*§. **Clinical Immunohaematology Lab; †Tumour Immunology Lab, Queensland Institute of Medical Research; §Department of Haematology, Princess Alexandra Hospital, Brisbane, QLD; ‡Department of Haematology, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia.*

We, as well as other investigators, have shown a critical pathogenetic link between Epstein-Barr virus (EBV) and the development of a range of malignant lymphomas, including Hodgkin, diffuse large B-cell lymphoma, post-transplant lymphoproliferative disorder (PTLD), and extranodal NK/T-cell lymphoma. These occur in the immunosuppressed and the "overtly" immunocompetent lymphomas that show a selective impairment of EBV immunity. The presence of EBV within the lymphoma cell is an adverse prognosticator but is also a potential

target. Restoration of EBV-specific T-cell immunity is an attractive therapeutic option. We hypothesize that adoptive immunotherapy of clinical grade EBV EBNA1-LMP1/2-specific T cells for relapsed/refractory EBV-positive lymphomas is safe resulting in the reconstitution of antiviral immunity and the induction of tumor lysis. We have recently commenced an Australia National Health and Medical Research Council phase I clinical trial of adoptive immunotherapy of in vitro expanded, EBV-specific cytotoxic T lymphocytes (CTLs) in EBV-positive lymphomas. This uses a novel replication-deficient adenoviral construct (''AdE1-LMPpoly'') that encodes specific EBV proteins expressed by all latency II, III EBV-positive lymphomas. CTLs are generated in the Queensland Institute of Medical Research's cGMP licensed facility. Earlier methodology was slow and technically demanding resulting in CTLs with minimal EBNA1 specificity. Our approach is a new technology that circumvents these limitations using a highly efficient, but relatively brief and autologous EBV-specific CTL expansion protocol. To date, 4 patients have been enrolled (2 lymphomatoid granulomatosis, 1 Hodgkin, 1 diffuse large B-cell lymphoma). In all the cases, clinical-grade EBV-specific CTLs were generated. CTLs were reinfused in both lymphomatoid granulomatosis patients. In the first patient, a short-lived remission was induced including complete eradication of skin lesions with demonstrable antiviral efficacy. However, subsequent relapse of disease occurred, but interestingly, there was no return of the skin disease. In the second patient remission was induced and is still ongoing. Our data, although preliminary, indicate that this approach is potentially feasible, safe, and efficacious. The trial is ongoing and aims to recruit a further 16 patients.

Engineered T Cells for Cancer Therapy

Carl H. June. Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA.

Although there are exciting examples of successful clinical strategies to mobilize the immune system to attack cancer cells, overall the results have been disappointing in randomized clinical trials. We are exploring the use of engineered T cells bearing chimeric receptors, strategies to augment their antitumor efficacy in adoptive transfer settings. The surface membrane glycoprotein, mesothelin, is a promising target for the immunotherapy of mesothelioma, ovarian, and pancreatic tumors because of the uniform overexpression of mesothelin, the benign phenotype of mesothelin null mice. We hypothesize that earlier trials of adoptive immunotherapy for cancer that have used cytotoxic T lymphocytes have failed because of poor trafficking to sites of tumor and insufficient effector functions to self-antigens. Our preclinical data indicate that the use of lentiviral engineered T cells with chimeric receptors that incorporate a ''tumor resistance genotype'' should have improved function for cancer immunotherapy. We have tested mesothelin-redirected T cells in humanized mouse models bearing tumor xenografts. The T cells are able to eradicate large, well-established tumors at an in vivo E:T ratio of at least 1:70. As a complementary strategy, we have engineered artificial antigen-presenting cells to express ligands for either CD28 or ICOS. These artificial antigen-presenting cells seem useful in reprogramming T cells and increasing the antitumor efficacy of adoptively transferred T cells. In ongoing clinical trials testing adoptive transfer of T cells after retroviral or lentiviral gene transfer, we find that[1] the T cells engraft and persist at high levels for 10 years or more indicating that central memory T cells with ''stem cell-like qualities'' can be transduced,[2] rectal mucosal biopsy studies taken from patients after adoptive transfer indicate that the T cells traffic with high efficiency to intra-epithelial lymphocytes. Finally, our preclinical studies with Jakobsen testing for T-cell receptors engineered for high affinity indicate the ability to ''convert'' polyclonal T cells to monoclonal T cells with potent redirected specificity for surrogate antigens, suggesting that tumor antigens for which a substantial repertoire of limitations in the natural pool of available T cells can be targeted with the adoptive transfer of engineered T cells.

References:

1. Carpenito C, et al. Control of large established tumor xenografts with genetically retargeted human T cells containing CD28, CD137 domains. *Proc Natl Acad Sci USA*. 2009;106:3360.
2. Milone MC, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells, increased antileukemic efficacy in vivo. *Mol Ther*. 2009.
3. Wang GP, et al. Analysis of Lentiviral Vector Integration in HIV+ Study Subjects Receiving Autologous Infusions of Gene Modified $CD4^+$ T Cells. *Mol Ther*. 2009.
4. Perez EE, et al. Establishment of HIV Resistance in CD4(+) T cells by Genome Editing using Zinc Finger Nucleases. *Nature Biotechnology*. 2008;26:808.
5. Varela Rohena A, et al. Control of HIV immune escape by CD8 T cells expressing enhanced T cell receptor. *Nature Med*. 2008;14:1390.
6. June CH. Principles of adoptive T cell cancer therapy. *J Clin Invest*. 2007;117:1204.

Genetically Engineered CD56-specific T Cells for the Treatment of Recurrent Neuroblastoma

Denise L. Kellar, Sonny Ang, Harjeet Singh, Lenka Hurton, Simon Olivares, Margaret J. Dawson, Matthew J. Figliola, Helen Huls, Dean A. Lee, Laurence J.N. Cooper. *Division of Pediatrics, U.T. M.D. Anderson Cancer Center, Houston, TX.*

Chimeric antigen receptors (CARs) combine the targeted specificity of a monoclonal antibody with the intracellular endodomains sufficient for T-cell activation and have been shown to be efficacious in both mouse experiments and clinical trials. Neuroblastoma (NBL) is the most common extracranial solid tumor in children, which is fatal in the majority of cases because of the widespread metastatic tumor burden, such as in the bone marrow. CD56 is a cell surface molecule expressed on nearly 100% of NBLs and several other cancers, thus making it a candidate antigen for targeting NBL. We hypothesized that the generation of a CD56-specific CAR (CD56RCD28) will redirect the specificity of T cells to $CD56^+$ NBL. The single-chain variable fragment from an anti-CD56 humanized murine monoclonal antibody, N901, was fused for CD3ζ, CD28 signaling endodomains to generate the CD56RCD28. The Sleeping Beauty transposon/transposase system for nonviral gene transfer was used to introduce the CD56RCD28

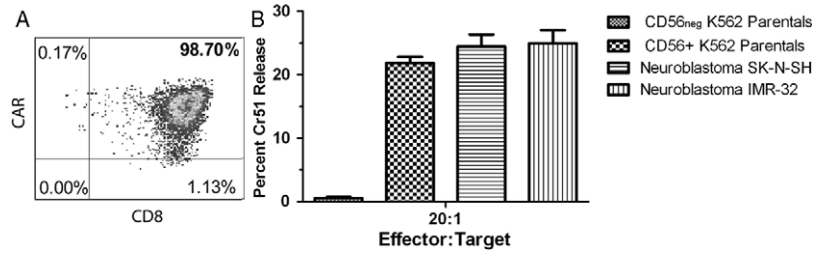

FIGURE 1 (Kellar). A, Phenotypic. B, Functional characterization of CD56-specific T cells.

CAR into primary human T cells. These genetically modified T cells were expanded in the presence of exogenous interleukin (IL)-2, IL-21 on K562 genetically modified to function as artificial antigen-presenting cells upon expressing the costimulatory molecules CD86, 4-1BBL, membrane-bound IL-15, and CD56 antigen. A population of CAR+ (predominantly $CD8^+$ T cells) was numerically expanded to clinically significant numbers, showed CD56-specific cytotoxicity (Fig. 1 previous page). The genetically modified T cells consisted of both effector, central memory subsets based upon CD62L, CD28, CD95 coexpression, consistent with the potential for sustained in vivo persistence. They also expressed high levels of granzyme-B and perforin-A supporting their cytotoxic potential, the CXCR4 chemokine receptor indicating their potential for in vivo homing to the bone marrow. These data show the ability to generate $CD56RCD28^+$ T cells with both effector and central memory phenotypes to clinically meaningful numbers using antigen-based artificial antigen presenting cell expansion. Future studies will address conditional activation of the CAR under hypoxia to limit CAR-dependent T-cell effector functions within the tumor microenvironment to attenuate deleterious off-target effects. Genetically engineered CAR+ T cells successfully target NBL in vitro establishing the potential for adoptive transfer of CD56-specific T cells for treatment of recurrent NBL.

The Impact of Clinical Parameters, in Cancer Patients Refractory to Standard Therapy, on the Feasibility of Expanding Tumor Antigen-specific T Cells Ex Vivo

J. Keller, Jennifer Childs, Yushe Dang, Lupe Salazar, Doreen Higgins, Nicole Bates, Andrew Coveler, Mary Disis. *Tumor Vaccine Group, University of Washington, Seattle, WA.*

Adoptive T-cell therapy is a treatment modality that has shown promise in patients with advanced stage melanoma. The application of T-cell therapy to patients with other solid tumor types, however, has not been well described. Two potential hurdles to successful tumor antigen-specific T-cell expansions in these patients are (1) the lack of accessible tumor-infiltrating lymphocytes (TILs) requiring the use of peripheral blood mononuclear cells as a source of T cells and, (2) such patients, who often receive years of chemotherapy before developing refractory disease, have many treatment options, and thus may have reduced ability to generate antigen-specific T cells. We have eralier reported results of a phase I clinical trial of adoptive T-cell therapy in patients with refractory breast or ovarian cancer. After vaccine priming, HER2-specific T cells were expanded for each patient, the number of antigen-specific T cells was augmented in vivo in some patients. Both, expansions ex vivo and augmentation of T cells in vivo, were heterogeneic among patients. We questioned whether any clinical parameter was responsible for the heterogeneity that was observed ($n = 8$). The clinical parameters that were evaluated included total white blood cell count, lymphocyte count at the time of cell collection, level of HER2 protein overexpression in the primary tumor or disease recurrence, time from last chemotherapy or last dose of trastuzumab, total number of earlier chemotherapy regimens, time from original diagnosis, from metastatic diagnosis, or from last HER2 vaccine, storage time of cell product before expansion, and patient age. Fold expansions of T cells ex vivo as well as the number of HER2-specific T cells generated in the product before infusion were not impacted by any of the clinical parameters assessed ($P > 0.05$ for all). When evaluating the augmentation and the persistence of the T cells in vivo, however, several clinical parameters seemed to influence antigen-specific T-cell expansion and persistence. The longer the interval from metastatic diagnosis ($P = 0.013$), the more chemotherapy regimens received ($P = 0.056$), and the greater the maximum number of HER2-specific T cells achieved in vivo. The elapsed time since vaccination also positively correlated with maximal in vivo HER2-specific T-cell levels ($P = 0.075$). However, these factors did not influence T-cell longevity in vivo ($P = 0.10$; $P = 0.46$; $P = 0.15$). Both lymphocyte count ($P = 0.075$) and white cell count ($P = 0.055$) positively correlated with T-cell persistence in vivo. These preliminary data suggest that clinical parameters associated with standard treatments may impact the ability to expand tumor antigen-specific T cells ex vivo and/or modulate T-cell expansion and persistence in vivo.

T-cell Delivery of Interleukin-12 to the Tumor Microenvironment Triggers Potent Endogenous Antitumor Responses

*Sid P. Kerkar**, Pawel Muranski*, Andrea Boni*, Andrew Kaiser*, Luis Sanchez-Perez*, Lydie Cassard*, Luca Gattinoni*, Douglas Palmer*, Zhiya Yu*, Yun Ji*, Madhu Sukumar*, Robert Reger*, Lindsay Garvin*, Ling Zhang*, Richard A. Morgan*, Giorgio Trinchieri†, Steven A. Rosenberg*, Nicholas P. Restifo*. **Surgery Branch, Center for Cancer Research, Bethesda;* †*Cancer and Inflammation Program, Center for Cancer Research, Frederick, MD.*

Interleukin-12 (IL-12) is a well-studied heterodimeric cytokine playing a central role in bridging both innate and cell-mediated immunity. Current treatments with IL-12 have not led to robust antitumor responses, but the effect of delivering IL-12 directly into the tumor microenvironment through a systemic approach is unknown. $CD8^+$ pmel-1 T cells specific for the melanoma-associated antigen, glycoprotein 100 (gp100), were transduced with a retroviral vector expressing the p40 and p35 subunits of IL-12 as a single functional molecule and transferred into B16-melanoma-bearing mice after nonmyeloablating total body irradiation (5 Gy). pmel-1 cells transduced with IL-12 caused regression of large established melanomas with 100-fold fewer cells than nontransduced cells without the need for systemic IL-2 or a gp100 encoding vaccine. T-cell receptor specificity allowed for delivery of IL-12 directly into the tumor microenvironment. Open repertoire $CD8^+$ cells transduced with IL-12 did not induce tumor regression whereas cells double-transduced with IL-12 and pmel-1 T-cell receptor reproduced a potent antitumor response. Using IL-12Rβ2−/− mice, we determined that the therapeutic effect was critically dependent on the ability of the host to respond to secreted IL-12 and not due to the enhanced functional quality of transferred cells. These findings reveal a key approach to the systemic localization of IL-12 to the tumor site and have major implications for improving future adoptive cell therapies.

Programming Tumor-reactive Effector Memory $CD8^+$ T Cells In Vitro Obviates the Requirement For In Vivo Vaccination

*Christopher A. Klebanoff**†, Zhiya Yu*, Leroy N. Hwang*, Douglas C. Palmer*, Luca Gattinoni*, Nicholas P. Restifo*. **Center for Cancer Research, National Cancer Institute/National Institutes of Health;* †*National Institutes of Health Research Scholars Program, HHMI, Bethesda, MD.*

$CD8^+$ T cells can undergo programmed activation and expansion in response to a short T-cell receptor stimulus, but whether in vitro programming can substitute for in vivo antigen stimulation is unknown. We show that self/tumor-reactive effector memory $CD8^+$ T cells programmed in vitro either with peptide-pulsed antigen-presenting cells or plate bound anti-CD3/anti-CD28 embark on a highly stereotyped response of in vivo clonal expansion and tumor destruction nearly identical to that of vaccine-stimulated tumor-reactive effector memory cells. The programmed response was associated with an interval of antigen-independent interferon-γ release that facilitated the dynamic expression of the major histocompatibility complex class I restriction element H-2Db on responding tumor cells, leading to recognition and subsequent tumor lysis. Further, the transfer of programmed cells genetically deficient in interferon-γ or a delay in the transfer of programmed cells more than 24 hours after stimulation entirely abrogated the benefit of in vitro programming. These findings extend the phenomenon of a programmable effector response in $CD8^+$ T cells to a therapeutic intervention relevant to current adoptive cell transfer protocols.

Labeling of Dendritic Cells and Lymphocytes With Iron Oxide MR Contrast Agents for Cellular MRI

*Yasunobu Kobayashi**†, Koichi Shimizu*†‡, Satoshi Ohno†§, Ryuji Okuyama†,‖, Kenichiro Imai†,‖, Valentina V. Ostapenko†, Tamiyo Kobayashi¶, Atsushi Aruga†‖#, Keishi Tanigawa*†. **J.B.Therapeutics, Inc; † Bio-Thera Clinic; ‡Shin-Itabashi Clinic; §IREIIMS, Tokyo Women's Medical University; ‖Institute of Gastroenterology, Tokyo Women's Medical University; ¶Olympus Corporation; #CICT, Tokyo Women's Medical University, Tokyo, Japan.*

Recent studies have shown that superparamagnetic iron oxide (SPIO) is a useful agent for labeling mammalian cells for noninvasive imaging of their migration and biodistribution. In this study, we tried to establishm optimal conditions for the labeling of monocyte-derived dendritic cells (Mo-DCs) and CD3-activated T (CAT) cells with clinically approved SPIO (Resovist). Efficient uptake and labeling of Mo-DCs with SPIO, probably through receptor-mediated endocytosis, were observed when SPIO was added into the culture medium in the early phase of Mo-DCs induction. Flow cytometry analysis revealed expression levels of CD80, CD83, CD86, CCR7, and HLA-DR in SPIO-treated Mo-DCs were similar to those in untreated Mo-DCs. In addition, we found that several functions of Mo-DCs such as antigen uptake, processing, and presentation were not affected by SPIO treatment. Compared with Mo-DCs, CAT cells showed lower ability of SPIO uptake during the whole period of culture. Although prolonged incubation and/or increasing concentrations of SPIO seemed to improve labeling efficiency, only a small number of T cells, which is not all T cells in CAT cells, were labeled with SPIO. SPIO-labeled CAT cells, however, were enriched by using magnetic cell separation devices, and cell population (eg, percentage of T cells and CD4/CD8 ratio) of these labeled CAT cells was quite similar to that of unlabeled CAT cells, suggesting that SPIO treatment did not affect the growth of CAT cells. Taken together, we conclude that the simple addition of SPIO in the culture medium makes it possible to label both Mo-DCs and CAT cells sufficiently and efficiently, and thus it could be one of the useful methods for monitoring the trafficking and biodistribution of these cells in clinical studies.

Genetically Engineered Malignant Glioma-specific T Cells

*Seogkyoung Kong**, Richard P. Junghans*, Saryn Doucette†, Prakash Sampath*. **Neurosurgery, Boston University School of Medicine & Brain Tumor Laboratory, Division of Surgical Research; † Pathology, Roger Williams Medical Center, Providence, RI.*

Malignant glioma (MG) is a devastating primary brain tumor for which there is no effective therapy to specifically target tumor cells. Targeted immunogene therapy has been shown to be effective in a number of tumor models without significant toxicity. We aim to develop potent MG-specific T cells as a highly innovative and very unique targeted immunogene therapy. Its appeal is in being able to use tumor-specific T cells that specifically target tumor cells that express tumor-associated antigens, leaving surrounding brain tissue relatively unscathed. We exploit interleukin (IL)-13Rα2 as an MG-specific tumor antigen due to its frequent overexpression on a majority of MG but not on normal brain tissues. Targeting IL13Rα2 on MG has a strong rationale supported by clinical development of IL13 immunotoxin molecules. Earlier studies showed that modifications of IL13 at 4 sites (Glu-13, Lys-105, Lys-106, or Arg-109) can neutralize or dramatically increase the affinity of IL13 to IL13Rα2, compared with the shared IL13Rα1, which is expressed on the normal brain cells and other tissues. The uniqueness of our approach is the following: (1) the CD28 costimulation that will lead to improved T-cell survival, activation, and tumor cell killing; (2) mutant IL13 designer T cells that show specific targeting and selectively enhanced cytotoxicity of IL13Rα2+ MG cells; and (3) innovative modification to coexpress miRNA specific for the human glucocorticoid receptor gene (miR-hGR) to overcome glucocorticoid-mediated immunosuppression. This has clinical significance because many glioma patients who might potentially be candidates for eventual clinical use require glucocorticoid treatment for control of peritumoral edema. We modified T cells using retroviral gene transfer to express a chimeric immune receptor comprising an extracellular high-affinity mutant IL13.E11K.R107K (both glutamic acid at position 11 and arginine at 107 changed to lysine) molecule linked to intracellular signaling components from CD3 ζ chain and CD28 costimulatory molecule. The IL13.E11K.R107K designer T cells show specific targeting and selectively enhanced cytotoxicity of IL13Rα2+ glioblastoma cells, and inhibit tumor growth employing a glioma xenograft model established with human glioma cells in athymic nude rats. Preliminarily, glucocorticoid-resistant IL13.E11K.R107K miR-hGR designer T cells show specifically enhanced cytotoxicity of IL13Rα2+ glioblastoma cells versus control designer T cells in the presence of corticosteroid dexamethasone. The IL13.E11K.R107K miR-hGR designer T cells have significant potential for the treatment of recurrent MG.

Antigen-specific T Cells Cultured in IL-21 Demonstrate Superior Antitumor Efficacy Secondary to the Secretion of IL-17 And TNF-α

Vy P. Lai, Ekram Gad, Laleh Bigdeli, Yushe Dang, Cleo Stannard, Mary L. Disis. *University of Washington, Seattle, WA.*

Adoptive T-cell therapy has been evaluated for the treatment of cancer and has shown promising, yet, limited clinical success. Methods to optimize the expansion of therapeutically effective tumor antigen (Ag)-specific T cells from the peripheral blood of cancer patients are needed. Vaccine priming before expansion has been shown to greatly facilitate ex vivo expansion from peripheral blood mononuclear cells. We questioned whether alteration of cytokine growth factors used to culture vaccine-primed T cells could impact the number, phenotype, and function of tumor Ag-specific T cells. We evaluated the culture of neu p101 (Class II peptide)-specific T cells with interleukin (IL)-2, IL-2+IL-4, IL-2+IL-7, IL-2+IL-12, IL-2+IL-15, IL-2+IL-18, IL-2+IL-21, and IL-2+IL-7+IL-15 (a combination of cytokines widely used for murine T-cell expansion). Culture conditions resulted in heterogenic cell expansions ranging from 1-fold to 2-fold depending on the cytokine mix. Moreover, the frequency of Ag-specific T cells was also variable in culture ranging from 1:4800 to 1:50,000. All cell cultures equally produced high levels of interferon-γ ($P > 0.05$). Only those cells cultured with IL-2+IL-21, however, secreted significant levels of IL-17 and tumor necrosis factor (TNF)-α compared with other combinations ($P = 0.014$ to $P = 0.05$). For in vivo functional studies, each cultured T-cell line was admixed with live mouse mammary carcinoma cells and implanted subcutaneously into the flank of TgMMTV-neu mice. The ability of the T cells to inhibit tumor growth at the local site was assessed. IL-2+IL-21 T cells showed significant tumor inhibition compared with the rest of the p101 specific T-cell cultures ($P = 0.003$ to $P = 0.049$). Various cytokine cultured T cells were also injected intravenously into TgMMTV-neu mice bearing spontaneous mammary tumors. Here, IL-2+IL-21 expanded p101-specific T cells, IL-2 expanded T cells, IL-2+IL-7+IL-15 expanded T cells, and naive T cells were compared for their ability to inhibit growth of established tumors. IL-2+IL-21 cultured T cells were again superior, significantly inhibiting tumor growth compared with IL-2 cultured cells ($P = 0.025$), IL-2+IL-7+IL-15 cultured T cells ($P = 0.038$), and naive T cells ($P = 0.022$). Blockade of IL-17 or TNF-α after adoptive T-cell therapy via neutralizing antibodies completely abrogated the in vivo efficacy of p101-specific IL-2+IL-21 cultured T cells. Studies are ongoing to determine the mechanism by which IL-17 and TNF-α mediate tumor regression after adoptive T-cell transfer.

IL-15 is Superior to IL-2 in Supporting Antigen-specific Expansion and Maintenance of T-cell Memory in Melanoma $CD8^+$ Tumor-infiltrating Lymphocytes

Yufeng Li, Jessica Hernandez, Richard Wu, Patrick Hwu, Laszlo Radvanyi. *Melanoma, MDACC, Houston, TX.*

The success of adoptive T-cell therapy (ACT) using expanded tumor-infiltrating lymphocytes (TILs) for metastatic melanoma relies on the ability of tumor antigen-specific T cells to persist and maintain antitumor effector function in the long term. A number of studies have reported a correlation between persistence of key antitumor TIL clonotypes and a favorable clinical response during ACT. Current research is examining more closely the phenotype and function of these persisting TILs and the culture conditions needed to generate TILs with improved persistence after the adoptive transfer. Interleukin (IL)-2 was approved by the Food and Drug Administration more than a decade ago and has been used both as single agent and in ACT for the treatment of metastatic melanoma. Both IL-2 and IL-15 share a common receptor γ chain. IL-2 stimulates $CD8^+$ and $CD4^+$ effector T cells as well as the expansion of $CD4^+$ regulatory T cells. IL-15 has more potent effects in supporting long-term expansion and homeostatic proliferation of memory $CD8^+$ T cells. IL-2, instead, drives $CD8^+$ T cells harder toward a terminally differentiated phenotype.

In this study, we compared the effects of IL-2 and IL-15 in supporting the long-term expansion (> 4 wk) of MART-1 + $CD8^+$ TILs after stimulation with MART-1 peptide-pulsed antigen-presenting cells in vitro. We found that IL-15 was superior to IL-2 in supporting the long-term survival and expansion of MART-1 + $CD8^+$ TILs after stimulation. Overall, IL-15 improved MART-1 + $CD8^+$ TIL yields by > 3-folds relative to IL-2. In addition, we found that both GrB expression, and antigen-specific cytotoxic T lymphocyte function improved the condition of IL-15. In particular, we found that IL-15 supported the expansion of the $CD27^+$MART-1 + $CD8^+$ T-cell subset better than IL-2. Moreover, MART-1 + $CD8^+$ T cells that maintained CD27 expression after IL-15 – induced expansion over the initial 28 days could be restimulated and expanded even further. Interestingly, we also found that IL-15 in some cases also induced increased cell surface CD28 expression during the 28-day culture period after antigenic stimulation, whereas IL-2 down-modulated CD28. These results indicate that IL-15 supports the persistence of melanoma antigen-specific effector-memory T cells and is superior to IL-2 in supporting TIL expansion and function in vivo during ACT. IL-15 may facilitate the maintenance of persistent effector-memory T cells after multiple contacts with the antigen in vivo and, in addition, may maintain critical levels of cytolytic molecule expression facilitating antitumor cytotoxic T lymphocyte activity in the long term. Thus, a switch to IL-15 cotherapy from IL-2, and/or augmenting endogenous IL-15 levels in vivo, may greatly improve the efficacy of ACT.

Using 19F MRI for In Vivo Tracking of Therapeutic Cells

*Robbie B. Mailliard**, Brooke M. Helfer*, Jelena M. Janjic†, Pawel Kalinski‡, Eric T. Ahrens†. **Research and Development, Celsense Inc.;* †*Biological Sciences, Carnegie Mellon University;* ‡*Department of Surgery, University of Pittsburgh, Pittsburgh, PA.*

Natural killer (NK) cells, T cells, and dendritic cells (DCs) are a few of the cell types being used as anticancer cellular therapeutics. A current limitation of these cellular therapies is the inability to track the cells once they are administered into a patient. Here we use a novel perfluorocarbon tracer agent to label, ex vivo, clinically relevant human immune cell types for the purpose of tracking the cells in vivo using 19F magnetic resonance imaging (MRI). Due to the minimal fluorine content in mammalian tissues, the 19F-labeled cells can be unambiguously detected, thus having an advantage over conventional metal ion-based contrast agents that rely on conventional proton (1H) MRI. The pairing of the 19F MR image with conventional 1H MRI, taken during the same MRI session, provides clear cell localization within the anatomic context. Here we show that both phagocytic and nonphagocytic cell types, including human NK cells, T cells, and DCs, can be effectively labeled without the inclusion of a transfection reagent and without impact on cell viability or function. Labeling efficiency was determined by performing NMR analysis on labeled cell pellets. As a proof of the principle, MRI efficacy was shown after the inoculation of perfluorocarbon-labeled human DCs into nonobese diabetic-severe combined immune deficiency mice. These DCs were successfully detected in vivo using 19F MRI, with a portion of the mature DCs selectively appearing in the draining lymph node region within 18 hours. This report provides support for the use of this noninvasive 19F MRI-based cell tracking technology as a clinical tool for monitoring therapeutic cells in vivo.

Transduction of Tumor-specific T Cells With the Gene Encoding CXCR2 Improves Migration to Tumor and In Vivo Antitumor Immune Responses

*Weiyi Peng**, Yang Ye*, Brian A. Rabinobich†, Chenwen Liu*, Yanyan Lou*, Minying Zhang*, Greg Lizee*, Patrick Hwu*. **Melanoma Medical Oncology;* †*Experimental Diagnostic Imaging, University of Texas MD Anderson Cancer Center, Houston, TX.*

Adoptive T-cell therapy (ACT) is a promising therapeutic modality for cancer. Although tumor regression can be dramatic in some ACT-treated patients, other patients do not experience any clinical responses. We hypothesize that one of the rate-limiting steps in ACT is the inefficient migration of T cells to tumors. Chemokines are secreted proteins, which are essential for mediating the trafficking of immune cells toward tumor sites. Melanomas specifically express the chemokines CXCL1 and CXCL8. However, we showed earlier that tumor-specific T cells fail to express the receptors for these chemokines such as CXCR2. Therefore, we hypothesized that the migration of T cells to tumors could be improved through the expression of the CXCR2 gene in T cells. In this study, we used transgenic pmel T cells, which recognize melanoma antigen gp100, and transduced these cells with our luciferase gene modified earlier. To visualize luciferase-expressing T cells within a tumor, a nonpigmented tumor is required. Therefore, we used the MC38 tumor model, which naturally expresses CXCL1. MC38 was transduced to express gp100 so that the cells could be recognized by pmel T cells. Mice bearing MC38/gp100 tumor cells treated with CXCR2/luciferase-transduced pmel T cells showed enhanced tumor regression and survival compared with mice receiving control luciferase-transduced pmel T cells (median tumor size 29.12 vs. 61.1 mm^2 on day 24 after tumor inoculation, respectively, $P < 0.001$). We also observed preferential accumulation of CXCR2-expressing pmel T cells in the tumor sites of these mice using bioluminescence imaging (median luciferase output of 191901 vs. 83480 photons/s/cm^2/sr, respectively on day 6 after T-cell transfer, $P = 0.024$). To confirm these results, we used B16 melanoma cells, which naturally express gp100, but do not express CXCL1. A similar enhancement in tumor regression and survival was observed when CXCR2-transduced pmel T cells were transferred into mice bearing CXCL1-transduced B16 tumors compared with mice treated with control pmel T cells (median tumor size 27.37 vs. 95.76 mm^2 on day 18 after tumor inoculation, respectively, $P < 0.001$). On the basis of these results, we conclude that the introduction of the CXCR2 gene into tumor-specific T cells can enhance their localization to tumors to mediate improved antitumor immune responses. This study may provide an important new avenue in "personalized cancer therapy" based on the chemokine profile of specific tumors, and plans are underway to translate this strategy to the clinic.

What are the Functional and Phenotypic Qualities of Therapeutically Successful Antitumor T Cells?

*Nicholas P. Restifo**, Christopher A. Klebanoff*†, Christian S. Hinrichs*, Pawel Muranski*, Luca Gattinoni*. **National Cancer Institute;* †*Howard Hughes Medical Institute, Bethesda, MD.*

We have described earlier how vaccination and lymphodepletion can augment the therapeutic effectiveness of naturally occurring or

genetically engineered tumor-specific T cells on adoptive transfer. Immunoablation using chemotherapy and total body irradiation enhances adoptive immunotherapy by liberating Toll-like receptor agonists from commensal gut bacteria and by eliminating T regulatory cells, myeloid-derived suppressor cells, and ''sinks'' for homeostatic cytokines.

Our current study is focused on elucidating the functional and phenotypic qualities of adoptively transferred T cells that are associated with tumor rejection. We have observed that the state of differentiation of antitumor T cells before their adoptive transfer is critically important for their effectiveness. Until recently, it was thought that the optimal antitumor $CD8^+$ T cells would be those that were highly cytolytic and capable of releasing large amounts of interferon-γ upon encounter with tumor cell targets when tested before adoptive transfer. However, it seems clear now that cytotoxic T cells represent a terminal, proapoptotic differentiation state of $CD8^+$ T cells. Paradoxically, the acquisition of full effector function in vitro impairs the in vivo antitumor efficacy of adoptively transferred $CD8^+$ T cells.[1] Central memory $CD8^+$ T cells [eg, those grown in interleukin (IL)-15] confer superior antitumor immunity compared with effector-memory T cells expanded using IL-2.[2,3] Furthermore, naive cells or $CD8^+$ T memory stem cells activated in the presence of IL-21 or Wnt, which are even less mature, are still more effective than central memory cells.[4,5] Thus, the state of maturation of $CD8^+$ T cells before adoptive transfer is inversely correlated with their effectiveness in vivo: ''younger'' T cells are better. It is important to note that undifferentiated T cells must be capable of maturing into fully functional T cells after transfer. This in vivo activation and differentiation of early $CD8^+$ T cell subsets can be enhanced by homeostatic cytokines and encounter with the cognate antigen.[6]

T-cell differentiation is also critically important for antitumor $CD4^+$ T cells, which can be robustly polarized in vitro before transfer. This polarization can skew their expression of transcription factors, cytokines, chemokines, and cell surface markers. $CD4^+$ T cells that are polarized to produce IL-17 (T_H17 cells) have increased effectiveness in antitumor models when compared with other T-cell subsets that have been tested.[7] Importantly, these T_H17 cells evolve after transfer into an immunoablated host into cells that are capable of producing interferon-γ. Thus, the differentiation states of antitumor $CD8^+$ and $CD4^+$ T cells are critical determinants of their effectiveness in vivo. These studies are consistent with data from human studies that indicate that young/undifferentiated T cells have increased effectiveness in the adoptive immunotherapy of melanoma and point the way toward the use of appropriately polarized $CD4^+$ T cells in patients with cancer.

References:

1. Gattinoni. *J Clin Invest*. 2005.
2. Klebanoff. *Proc Natl Acad Sci*, 2004.
3. Klebanoff. *Proc Natl Acad Sci*. 2005.
4. Hinrichs. *Blood*. 2008.
5. Gattinoni. *Nat Med*. 2009.
6. Gattinoni. *Nat Rev Immunol*. 2006.
7. Muranski. *Blood*. 2008.

Cell Transfer Therapy for Patients With Metastatic Cancer

Steven A. Rosenberg. Surgery Branch, National Institutes of Health, Bethesda, MD.

Adoptive cell transfer (ACT) therapy refers to a treatment approach in which lymphocytes with antitumor activity are transferred to the tumor-bearing host with the aim of mediating tumor regression. ACT represents the best available treatment for patients with metastatic melanoma and can mediate objective responses (RECIST criteria) in up to 72% of patients.

We have conducted a series of 3 consecutive clinical protocols in patients with metastatic melanoma using autologous tumor-infiltrating lymphocytes (TILs) that are grown ex vivo from resected lesions and reinfused into the autologous patient along with interleukin-2 (IL-2). In the first of these trials, 43 patients were treated with autologous TIL transfer after a nonmyeloablative chemotherapy consisting of cyclophosphamide (60 mg/kg for 2 days) and fludarabine (25 mg/m^2 for 5 days). In the second and third clinical trials, this chemotherapy was given in conjunction with either 200 cGy or 1200 cGy. The objective response rates (by RECIST criteria) in these 3 trials were 49%, 52%, and 72%. In the latter trial, 7 patients (28%) were complete responders. Durable responses have been seen at all sites including lung, liver, brain, bone, lymph nodes, and subcutaneous tissues. Twelve of the 13 complete responses in the 3 trials are ongoing between 21 and 26 months. Forty-two of the 53 responding patients had received prior interleukin-2 and many had received prior chemotherapy as well. The actuarial 3-year survival for patients receiving the chemo-radiation preparative regimen was 44%. Thus, T-cell – based immunotherapy is capable of mediating the regression of large vascularized invasive metastatic melanoma in humans. The widely held belief that immunotherapy can only affect minimal disease in the adjuvant setting is certainly not the case.

There is a significant association between persistence of the transferred cells in the circulation at 1 month and the likelihood of achieving a clinical response ($P < 0.001$). Cells with longer telomeres ($P < 0.01$) and with a higher percentage of cells expressing CD27 ($P < 0.001$) are also associated with objective clinical responses.

Some patients do not have easily harvestable lesions to provide a source for the TIL. In addition, TIL with antitumor activity can only reproducibly be obtained from patients with melanoma and not from other cancers. We have thus developed new approaches to ACT that involve the transduction of antitumor T-cell receptors into autologous circulating lymphocytes. This genetic engineering provides normal circulating peripheral lymphocytes with antitumor activity that is often as strong or stronger than TIL obtained by conventional methods. In current clinical trials of the adoptive transfer of these gene-modified cells, objective response rates are approximately 30%. We have now generated retroviruses that encode either conventional or chimeric T-cell receptors that can recognize antigens such as NY-ESO-1, CEA, and CD19 that are present on common cancers. Clinical trials using the adoptive transfer of these genetically engineered cells are now underway.

In summary, ACT can mediate the complete regression of metastatic melanomas in heavily pretreated patients. The ability to genetically engineer normal circulating lymphocytes to acquire antitumor properties provides an opportunity to extend this approach to patients with common epithelial tumors.

Impact of Bortezomib-induced Proteasome Inhibition on Antitumor T-cell Responses In Vivo

*Anil Shanker**†, Rachel De Kluyver*, John W. Wine*, Thomas J. Sayers*†. **NCI-Frederick; †SAIC-Frederick Inc, Laboratory of Experimental Immunology, Cancer and Inflammation Program, Frederick, MD.*

Inhibition of cellular proteasome function crucial for protein turnover is a recent approach to overcome the resistance of tumors to cell death, a major clinical problem. Bortezomib (also known as Velcade/PS-341), a specific and reversible proteasome inhibitor, has been shown to sensitize some mouse and human tumors to apoptosis induced by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or its receptor (DR5) agonist monoclonal antibody (mAb). In established mouse renal cell (Renca) and breast (4T1) tumor models, the administration of DR5 agonist mAb to tumor-bearing mice pretreated with bortezomib reduced experimental lung metastases. The combination of bortezomib and DR5 agonist mAb also significantly increased long-term survival of mice bearing Renca tumors compared with either agent alone. The therapeutic effects of bortezomib on tumor metastases and survival of tumor-bearing mice occurred in the absence of overt toxicity. However, little is known about the effects of bortezomib on specific immune responses. We thus analyzed the effects of proteasome inhibition on antitumor T-cell responses in vivo. In mice with established Renca- hemagglutinin (HA) tumor expressing influenza virus HA as a surrogate tumor antigen, we evaluated

the antigen-specific proliferation of adoptively transferred monoclonal HA-specific major histocompatibility complex class I or II restricted T cells following various schedules of bortezomib administration. No inhibition of T-cell proliferation or cytolytic function in vivo was observed after bortezomib treatments, nor was there any evidence of defective dendritic cell maturation in bortezomib-treated mice. However, a significant decrease in total T-cell yield was noted in the tumor-draining lymph nodes of mice treated with bortezomib. Thus, bortezomib-inhibitory effects on T-cell responses seem quantitative rather than qualitative. The basis for this decrease in T-cell number is under further investigation. The findings imply that, with appropriate scheduling, it may be possible to combine bortezomib-induced sensitization of tumors to apoptosis with tumor-specific T-cell immunotherapy regimens for increased therapeutic benefit in cancer. Funded by NCI Contract N01-CO-12400.

Combination Therapy With Antiangiogenic Agents and Adoptive Cell Therapy (ACT) in a Murine Tumor Model

Rajeev K. Shrimali, Marc R. Theoret, Zhiya Yu, Dhanalakshmi Chinnasamy, Nicholas P. Restifo, Steven A. Rosenberg. *Surgery Branch, NCI, NIH, Bethesda, MD.*

Antiangiogenic agents have been shown to mediate antitumor effects when administered with chemotherapy, possibly owing to stabilization of the tumor vasculature and increased exposure of tumor cells to circulating cytotoxic molecules. We have thus examined whether antiangiogenic agents such as antibodies to mouse vascular endothelial growth factor (VEGF) or VEGF receptor (VEGFR) could enhance the therapeutic impact of adoptively transferred cells in an ACT murine model of melanoma. Anti-VEGF antibody (Genentech, Inc) alone had minimal impact on the growth of established B16 tumors. When used in combination with anti-gp100 transgenic pmel-1 T cells, a highly significant B16 tumor treatment ($P = 0.009$) and prolonged median survival ($P = 0.003$) was seen when compared with ACT alone. Our preliminary cell infiltration studies showed a significant increase in pmel-1 T cells (Ly5.1 + VB13 +) infiltrating into B16 tumors after a single dose of 100 μg anti-VEGF compared with mice receiving ACT alone. DC101 antibody (anti-VEGFR2, Imclone systems) alone had minimal impact on the growth of established B16 tumors, and when used in combination with ACT had no significant effect on B16 tumor treatment and no effect on cell infiltration when compared to control groups receiving ACT alone. Thus, anti-VEGF antibody can increase the efficacy of ACT in this preclinical murine model. The application of this combination therapy for the treatment of human cancer is being planned.

High-Quality and High-Avidity T-cell Clones Specific for Tumor-associated Antigens and How to Find Them

*Susanne Wilde**, Slavoljub Milosevic*, Stefani Spranger*, Matthias Schiemann†, Dirk H. Busch†,‡, Bernhard Frankenberger*, Dolores J. Schendel*‡. **Institute of Molecular Immunology, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health;* †*Institute of Microbiology, Immunology and Hygiene, Technical University Munich;* ‡*Clinical Cooperation Group Immune Monitoring, Institute of Molecular Immunology, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Munich, Germany.*

As most tumor-associated antigens represent overexpressed self-proteins, T cells with high avidities have been eliminated during negative selection in the thymus to prevent autoimmunity. Therefore, we compared self-restricted and allo-restricted T-cell lines and clones with the aim of identifying properties that discriminate high-avidity from low-avidity T cells. Autologous $CD8^+$ T cells were primed using either dendritic cells of HLA-A2 − donors pulsed with RNA encoding the tumor-associated antigen tyrosinase plus allogeneic HLA-A2 RNA or dendritic cells of HLA-A2 + donors loaded with tyrosinase RNA alone. Multimer + $CD8^+$ cells were sorted and then cloned by limiting dilution, and uncloned cells were expanded as bulk lines using antigen-independent stimulation. As high-intensity multimer staining was earlier shown to indicate strong T-cell receptor (TCR)-ligand interactions, mean fluorescence intensities of multimer binding was used as a first estimate of structural TCR-MHC/peptide binding affinity. A second estimate was made on the basis of loss of multimer binding over time (ie, multimer off-rate), as a slower off-rate suggests that TCR-ligand interactions are more stable and of higher structural affinity. Differences were observed directly in primed cultures and after sorting of bulk lines of four donors. Multimer + $CD8^+$ cells of HLA-A2 + donors had lower MFI of multimer binding compared with HLA-A2 − donors, and percentages of double-positive cells were also lower. A more rapid loss of multimer binding was measured over time. These characteristics of multimer binding indicated that self-restricted T cells were of lower TCR affinity compared with allo-restricted T cells. At the clonal level, we observed more disparities with regard to the multimer analyses, as there was a trend but no significant correlation between multimer analysis and functional avidity, as measured by responses to titrated amounts of specific tyrosinase peptide pulsed on T2 cells. These results revealed that multimer MFI and off-rates alone do not conclusively predict functional avidity of T-cell clones.

Synergistic Killing of Tumor Cells by IL-2 − activated NK Cells and Tumor-specific CTLs

*Shaohong Yu**, Juliet Wynn*, Andrew Fischer*, Julia Rich*, Michael T. Lotze†, Per H. Basse*. **UPCI/Immunology;* †*UPCI/Surgery, University of Pittsburgh, Pittsburgh, PA.*

Tumor-specific cytotoxic T lymphocytes (CTLs) recognize major histocompatibility complex (MHC)-I − presented antigen by tumor cells to mediate effector function. In contrast, natural killer (NK) cells lack T-cell receptors and are thus not dependent on expression of specific antigens by tumor cells for recognition. Tumor cells with low MHC-I expression are, in general, lysed by NK cells to a greater degree than high MHC-I-expressing tumor cells. Heterogeneous antigen/MHC-I expression found in tumors suggests that coordinate activity between T and NK cells may be necessary. We studied the ability of mixed CTL/NK effector cell populations to recognize B16 and OVA-transfected B16 (M05) tumor cells.

While interleukin-2 − activated NK (A-NK) cells killed B16 and M05 cell equally well, in vitro activated CD8 cells from OT-I transgenic mice (specific for SIINFEKL presented in the context of H-2Kb) recognized, as expected, the M05 cells, but not the B16 cells. On a per cell basis, A-NK cells were more cytolytic than the CTLs. When A-NK cells and OT-1 CTLs were mixed at varying ratios, additive cytotoxicity was found, except at high CTL:A-NK ratios (around 100:1), where a reduction of cytotoxicity was often observed. As CTLs and A-NK cells do not kill each other when mixed, this reduction in cytotoxicity may be explained by physical hindrance of A-NK lysis in the presence of high numbers of the somewhat less cytotoxic CTLs. Interestingly, at more moderate CTL:A-NK ratios (around 2:1-5:1), the effect of mixing CTLs and A-NK cells on the resultant cytotoxicity was synergistic. M05 cells incubated overnight with A-NK cell-derived supernatants were killed more efficiently by the CTLs than control M05 cells. A substantial increase in MHC-I expression by M05 tumor cells incubated with A-NK cell supernatants was noted. As expected, these M05 cells were killed more efficiently by the CTLs than control M05 cells. These effects of the A-NK cell-derived supernatants were abolished by addition of blocking antibodies to interferon-γ. M05 lung tumors infiltrated by adoptively transferred, interferon-γ − producing A-NK cells expressed substantially higher levels of H-2 Kb than M05 lung tumors from control animals. Work is in progress to determine whether this increase in MHC expression will result in more efficient therapy mediated by adoptively transferred CTLs.

Thus, ex vivo propagated A-NK cells and tumor-specific CTLs function additively (and, at certain NK:CTL ratios, synergistically)

to kill tumor cells. Transfer of mixtures of A-NK cells and tumor-specific CTLs will likely be more efficient against tumors with variable MHC/antigen positivity of tumor cells than the adoptive transfer of either effector cell type alone.

CLINICAL TRIALS OF NEW AGENTS

Antitumor Effect of Concholepas Concholepas Hemocyanin (CCH) Using B16-F10 Mouse Melanoma Cells as a Model

*Sergio Arancibia**, Miguel Del Campo*, Alfredo De Ioannes†, María I. Becker*†. **Fundación Ciencia y Tecnología para el Desarrollo; †Research and Development, Biosonda Corporation, Santiago, Chile.*

Hemocyanins are giant glycoproteins present in the hemolymph of some mollusks and arthropods, which when inoculated in mammals act as potent immunostimulant inducing a strong T_H1 activation. This response, applied in bladder cancer therapy and in tumor lysate-pulsed dendritic cell vaccines, has been attributed in part to the carbohydrate moieties of these proteins; however, the molecular and cellular mechanisms explaining its immunostimulatory properties are still largely unknown.

Here we showed that the antitumor effect of Concholepas concholepas hemocyanin (CCH) is not restricted to bladder cancer therapy, as it can delay tumor progression in a B16-F10 mouse melanoma model. Flow cytometry analysis showed that CCH tagged with AlexaFluor488 is poorly endocytosed by B16-F10 cells, and MTT assays indicated that neither CCH nor KLH (keyhole limpet hemocyanin), in doses ranging between 50 and 200 μg/mL, has an impact on cell survival after 24 and/or 72 hours of culture in vitro. In addition, the incorporation of CCH did not increase the expression of major histocompatibility complex-I (H-2Kb) in tumor cells after 24 hours of treatment. We conclude that, in vitro, CCH and KLH per se did not affect the viability or induce cell death in this melanoma cell line.

In vivo experiments showed that CCH therapy, after the exposure to B16-F10 cells, significantly diminished the tumor volume and increased mouse survival in this orthotropic melanoma model (C57BL/6). At the same time, these effects are related to the rise in interferon-γ production on day 13 after the challenge with melanoma cells. Throughout the assays, neither allergic reactions nor toxic effects owing to CCH were observed.

This study supports for the first time possible applications of CCH as a nonspecific immunostimulant, especially as part of therapies for different types of cancer.

FONDECYT grant 1050150. S. Arancibia is a CONICYT doctoral fellow.

Evaluation of Immune and Clinical Responses in a Phase 1 Trial of Intranodal Naked DNA Priming and Peptide Boost With PRAME and PSMA (MKC1106 PP), in Patients With Antigen-positive Solid Tumors

Mihail Obrocea*, Nicholas J. Vogelzang†, Marc S. Ernstoff‡, Jeffrey Weber§, Lee Cranmer‖, John Marshall¶, Thomas Kundig#, Dar Rosario*, Zhiyong Qiu*, *Adrian Bot**. **MannKind Corp, Valencia, CA; †US Oncology, Las Vegas, NV; ‡Dartmouth-Hitchcock Medical Center, Lebanon, NH; §H. Lee Moffitt Cancer Center, Tampa, FL; ‖Arizona Cancer Center, Tucson, AZ; ¶Lombardi Comprehensive Cancer Center, Washington DC, DC; #University of Zurich, Zurich, Switzerland.*

Background: PRAME (preferential antigen of melanoma) and PSMA (prostate-specific membrane antigen) are potential targets for active immunotherapy in cancer. MKC1106-PP is an investigational agent consisting of 3 components: a plasmid (pPRA-PSM) and 2 peptide analogs (E-PRA and E-PSM).

Methods: The patients enrolled had advanced cancer, and were HLA-A2 and tumor antigen-positive. The plasmid dose for bilateral injection was fixed at 1200 μg/injection and 2 peptide doses were used: "low dose" of 22.5 and 30 μg, and "high dose" of 150 and 300 μg of peptide/injection, for E-PRA and E-PSM, respectively. All components were administered separately into lymph nodes under ultrasound guidance. Patients were evaluated clinically after 2 therapeutic cycles (12 wk). Those determined to be nonprogressors continued on therapy and could receive up to 6 cycles of treatment. Immune response was assessed by tetramer and enzyme-linked immunosorbent spot analysis.

Results: A total of 26 patients with various tumor types were dosed (13 in each dose cohort). The treatment regimen was safe and well tolerated. Seven patients showed evidence of clinical response and completed 4 or more cycles of therapy: 4 of 12 prostate carcinoma patients, both kidney cancer patients, and 1 of 10 melanoma patients. To date, the best clinical outcomes include a patient with prostate carcinoma with objective tumor regression and PSA decline (30+ wk); 2 patients with prostate carcinoma with stable disease (36+ wk) accompanied by PSA velocity change; a patient with kidney cancer with no evidence of disease, postresection, in a neoadjuvant setting (72+ wk); and a patient with metastatic melanoma (M1c stage) with stable disease at 72+ weeks. Twelve of 19 evaluable patients showed transient or persistent expansion of T-cells for the immunizing antigens, after treatment. The patients who showed evidence of clinical benefit showed early expansion of specific T-cells in the blood, in a larger proportion (5 out of 6 evaluable patients) compared with only 7/14 patients with disease progression.

Conclusions: Intranodal immunization against PRAME and PSMA induced immune responses and clinical benefit in patients with solid tumors with minimal toxicities, laying the foundation for phase II testing in select cancers.

Immune and Clinical Responses in a Phase I Trial of Intranodal Naked DNA Priming and Peptide Boost With MART-1 and Tyrosinase (MKC1106 MT), in Patients With Advanced Melanoma

Antoni Ribas*, Jeffrey Weber†, Mihail Obrocea‡, Thomas Kundig§, Dar Rosario‡, Zhiyong Qiu‡, *Adrian Bot‡*. **U.C.L.A., Los Angeles; ‡MannKind Corp, Valencia, CA; †H. Lee Moffitt Cancer Center, Tampa, FL; §University of Zurich, Zurich, Switzerland.*

Background: MKC1106-MT is an investigational immunotherapeutic regimen directed against the melanoma antigens MART-1/Melan-A and tyrosinase, delivered as a recombinant plasmid prime and a peptide boost, both by intralymph node administration. It results in a significant expansion of antigen-specific, effector $CD8^+$ cells and antitumor activity in animal models.

Methods: HLA*0201-positive patients with unresectable stage IIIc or IV melanoma were enrolled in 2 cohorts. Both cohorts received the same dose of plasmid (1200 μg/injection) on days 1, 4, 15, and 18 of each treatment cycle, followed by a dose escalation of peptide (100 μg or 300 μg) on days 29 and 32. All components were administered separately into inguinal or axillary lymph nodes under ultrasound guidance. Patients underwent immunologic and clinical evaluation every 6 weeks for up to 8 cycles (1 y). Immune responses were measured by MART-1/Melan-A and tyrosinase tetramer and direct enzyme-linked immunosorbent spot.

Results: In all, 18 patients were dosed, 7 in the low-dose peptide cohort and 11 in the high-dose peptide cohort, with 4 patients continuing on treatment. Ten patients (2 in the low-peptide dose cohort and 8 in the high-peptide dose cohort) completed 2 or more cycles of therapy. All patients coexpressed the target antigens in tumor biopsies. Treatment was well tolerated with occasional Grade 1 or 2 adverse events of pain in the injection area and fatigue. Three patients with M1a melanoma (1 in the low-peptide dose cohort and 2 in the high-peptide dose cohort) had partial responses by RECIST criteria, with 59%, 60%, and 30% objective tumor shrinkage. All are durable responses, ongoing at 48+, 36+, and 36+ weeks, respectively. In addition, 1 patient in the high-peptide dose cohort had stable disease for 18+ weeks, also ongoing. Six patients demonstrated expansion of Melan

A/MART-1 – specific $CD8^+$ T cells. Interestingly, in all patients with an objective clinical response a significant population of Melan A/MART-1 – specific T-cells was detectable at baseline. In addition, the patients with macroscopic metastatic disease confined to lymph nodes (M1a stage) had a higher likelihood of Melan A/MART-1 – specific T-cells at baseline, compared with patients with visceral metastases (M1b and M1c stage).
Conclusions: Intranodal immunization against Melan A/MART-1 and tyrosinase, with a naked DNA prime and a peptide boost, induces objective tumor responses in patients with advanced melanoma with minimal toxicities. Clinical development will continue in phase II testing.

Increased CD4 and CD8 Memory T-cell Proliferation After Anti-OX40 Administration to Cancer Patients: Immunologic Assessment of a Phase I Clinical Trial

Magdalena Kovacsovics-Bankowski, Edwin Walker, Kevin Floyd, Walter Urba, Brendan Curti, Andrew D. Weinberg. *Robert W. Franz Cancer Research Center, Earle Chiles Research Institute, Portland, OR.*

OX40, a member of the TNF superfamily, is a potent costimulatory molecule expressed transiently at the surface of CD4 and CD8 T cells. Stimulation of OX40 augments T-cell effector function and survival. OX40 is also expressed by $CD4^+$ $CD25^+$ T regulatory cells, and engagement of OX40 on these cells has been shown to abrogate their suppressive function. Several studies, performed in mice, have shown that OX40-triggering enhances antitumor immunity and tumor-free survival. These results have prompted the initiation of phase I clinical trial using a mouse antihuman OX40 agonistic antibody in patients diagnosed with tumor. The study was designed to include 10 patients per group with a dose escalation of 0.1, 0.4, and 2 mg/kg of anti-OX40 administered on Days 1, 3, and 5, respectively. Immune monitoring analysis, over a 2-month period, was performed on PBL by flow cytometry, using a 10-color antibody panel detecting CD3, CD4, CD8, CD95, CD28, CD25, CD127, CCR7, Foxp3, and Ki-67. In this abstract, we report the result of the first 2 cohorts: cohort 1 (n = 10) and cohort 2 (n = 8) that received 0.1 mg/kg and 0.4 mg/kg of antibody, respectively. The overall toxicity was low and partial tumor regression in 5 out of 20 patients was observed. The proliferation of memory T-cells (gated on $CD95^+$) was analyzed by intracellular staining with anti – Ki-67 antibody, and T regulatory cells were characterized by the coexpression of CD4 and Foxp-3. On Day 15, we observed a 2-fold to 3-fold increase in the proliferation of $CD4^+$ $CD95^+$, mostly in the FoxP3 – population. There was no significant increase in the turnover of $CD4^+$ $FoxP\text{-}3^+$ T regulatory cells over the time course of this study. The proportion of cycling $CD8^+$ $CD95^+$ T-cells also peaked 15 to 29 days after the administration of the antibody with a 2-fold to 4-fold increase of cycling cells compared with a group of 9 controls. We also observed on Day 15 an augmented proliferation (2-fold to 3-fold) of non-CD3 cells, mostly natural killer cells. These results suggest that the administration of an anti-OX40 antibody, in these patients, induces proliferation of $CD4^+$ T helper cells, $CD8^+$ T-cells, and natural killer cells in a dose-dependent manner. We are currently sorting $CD8^+$, Ki-67+ T-cells after patients received anti-OX40 antibody to determine whether they are enriched for tumor-reactive T-cells.

Elevated Angiotensin II Levels in Plasma and Malignant Effusions of Cancer Patients: Recombinant Human ACE2 as Novel Biological Cancer Therapy

*Hans Loibner**, Evelyne Janzek*, Bernhard Peball*, Romana Schaefer*, Manfred Schuster*, Thomas Bauernhofer†, Hellmut Samonigg†. **Apeiron Biologics, Vienna;* †*University Hospital, Graz, Austria.*

The membrane-anchored glycoprotein Angiotensin-converting enzyme 2 (ACE2) is the key counterbalancing enzyme of an activated Renin Angiotensin system (RAS), and is expressed in most tissues. ACE2 degrades Angiotensin II (Ang II) to yield Angiotensin (1-7) [Ang(1-7)]. Ang II, the key effector peptide of the RAS, increases blood pressure, but is also proinflammatory, angiogenic, a potent mitogen, and increases vascular permeability. In contrast, Ang(1-7) has vasodilatory, anti-inflammatory, anti-angiogenic, and antiproliferative properties. Thus, ACE2 acts in a counter-regulatory manner by shifting the balance between Ang II and Ang(1-7). There is increasing evidence that an activated RAS and elevated Ang II levels play a role in tumor development. For example, reduced ACE2 expression and elevated Ang II was found in human pancreatic cancer tissues. Ang(1-7) has been shown to retard tumor growth and reduce vascular endothelial growth factor levels in murine tumor models.
A novel biologic approach for cancer treatment may be based on the use of ACE2 to degrade elevated tumor-promoting Ang II levels to generate beneficial Ang(1-7). In this context, we are developing recombinant soluble ACE2 (rhACE2) for therapy of various diseases with an imbalanced RAS. rhACE2 (expressed in CHO cells and purified to homogeneity under cGMP) efficiently degrades Ang II to Ang(1-7) in vitro and in vivo. rhACE2 did not show any side effects or toxicities in preclinical toxicology studies in nonhuman primates.
To explore a possible role of elevated Ang II in formation of malignant effusions in cancer patients, we assess Ang II levels in plasma and in malignant effusions. In all, 11/19 patients (58%) present with elevated Ang II concentrations in plasma (in pts with elevated Ang II: median 75 pg/mL; mean 126 pg/mL, range 37.0-496.5 pg/mL). There is a significant correlation between the volume of malignant effusions and Ang II levels in plasma, indicating that elevated Ang II levels contribute to the formation and severity of effusions.
The assessment of Ang II levels in cancer patients is still ongoing; an update will be presented at the conference. Taking into account the excellent tolerability of rhACE2, it is justified to treat advanced cancer patients with malignant effusions and elevated Ang II levels with this enzyme. Pathologically elevated Ang II may be normalized, leading to beneficial therapeutic effects, such as decreased formation of effusion, as well as decreased proliferation of malignant cells, reduced inflammation, and reduced angiogenesis. A clinical study with rhACE2 in cancer patients with elevated Ang II levels is in progess.

IMP321 and Weekly Paclitaxel as First-line Chemoimmunotherapy for Metastatic Breast Cancer (MBC)

*Frederic Triebel**, Chrystelle Brignone*, Gutierrez Maya†. **Immutep S.A., Orsay;* †*Centre René Huguenin, Saint Cloud, France.*

Background: IMP321 (hLAG-3Ig), a novel immunomodulator:

1. Derived from the natural human protein LAG-3 (CD223), a ligand for major histocompatibility complex (MHC) class II molecules, in the form of a soluble fusion protein.
2. Acts indirectly on T-cell responses by MHC class II+ antigen-presenting cell activation.[1]
3. IMP321 induced both sustained CD8 T-cell activation and an increase in the percentage of long-lived effector-memory CD8 T cells in all patients at doses above 6 mg injected biweekly in metastatic renal cell carcinoma patients with improved progression-free survival.[2]
4. Chemoimmunotherapy protocol rationale: boosting the dendritic cell network when it is loaded with tumor antigens for several days after chemotherapy (secondary to tumor cell apoptosis) with repeated subcutaneous injection of the antigen-presenting cell-specific activator IMP321 may increase cytotoxic CD8 T-cell responses.

Methods: This is an open-label, fixed, dose-escalation phase I study, performed in an ambulatory setting in patients receiving first-line chemotherapy for metastatic breast cancer the standard 6 cycles of weekly paclitaxel (80 mg/m^2) at day 1, day 8, and day 15 of a

4-week cycle. Three IMP321 doses, 0.25, 1.25, and 6.25 mg given subcutaneously, were tested, given at day 2 and day 16 of this 4-week cycle, for 6 courses.

Results: IMP321 was very well tolerated. Thirty patients have been treated to date with no grade II/III adverse events related to the product. The interim results are based on tumor regression under RECIST criteria in the first 2 cohorts of 16 patients compared with the historical control group, which is the weekly paclitaxel arm of a recent randomized phase III study (*N Engl J Med*. 2007; 357:2666-76). They show a clinical response rate of 50% compared with 25% with paclitaxel alone ($P = 0.03$).

Conclusions: IMP321 in metastatic breast cancer patients treated with first-line chemotherapy was well tolerated when given subcutaneously over 6 months and enhanced significantly the overall response rate (interim analysis).

References:

1. Brignone C, Grygar C, Marcu M, et al. A soluble form of lymphocyte activation gene-3 (IMP321) induces activation of a large range of human effector cytotoxic cells. *J Immunol*. 2007;179:4202.
2. Brignone C, Escudier B, Grygar C, et al. A Phase I Pharmacokinetic and Biological Correlative Study of IMP321, a Novel MHC Class II Agonist, in Patients with Advanced Renal Cell Carcinoma. *Clin Cancer Res*. 2009. In press.

Apoptosis Regulator Protein PBF is an Immunologic Target for Patients With Osteosarcoma

*Tomohide Tsukahara**†, Satoshi Kawaguchi†, Toshihiko Torigoe*, Masanobu Kano*†, Satoshi Nagoya†, Mitsunori Kaya†, Takuro Wada†, Toshihiko Yamashita†, Noriyuki Sato*. *Departments of *Pathology; †Orthopaedic Surgery, Sapporo Medical University, Sapporo, Japan*.

To develop peptide-based immunotherapy for osteosarcoma, we earlier identified papillomavirus binding factor (PBF) as a cytotoxic T lymphocyte (CTL)-defined osteosarcoma antigen in the context of HLA-B55. PBF is an apoptosis regulator, and is associated with 14-3-3 and Scythe/BAT3. In this study, we discovered an HLA-A*0201-restricted epitope derived from PBF and characterized the peptide-specific CTLs in peripheral blood lymphocytes of HLA-A*0201+ patients with osteosarcoma. In accordance with the BIMAS score, we synthesized 10 peptides from the amino acid sequence of PBF. Subsequent screening with an HLA class I stabilization assay revealed that a PBF A2.2 peptide had the highest affinity to HLA-A*0201. $CD8^+$ T cells reacting with PBF A2.2 peptide were detected in 3 of 5 patients at frequencies from $2 \times 10\text{-}7$ to $5 \times 10\text{-}6$ using limiting dilution/mixed lymphocyte peptide culture followed by tetramer-based frequency analysis. Next, a tetramer-positive PBF A2.2-specific CTL line, 5A9, was obtained from one of the tetramer-positive CTL pools. CTL 5A9 specifically killed PBF+HLA-A2+ osteosarcoma cell lines, and T2 cells pulsed with PBF A2.2. Moreover, 5A9 killed autologous tumor cells. For the further characterization of the CTLs, we established tetramer-positive CTL clones derived from 5A9 using single-cell sorting with FACS AriaII. The resultant CTL clones also recognized PBF A2.2-pulsed T2 cells and PBF+HLA-A2+ osteosarcoma cell lines. These findings indicated that PBF A2.2 serves as a CTL epitope on osteosarcoma cells in the context of HLA-A2. We have begun a clinical phase I study of vaccination with PBF A2.2 for patients with osteosarcoma.

CYTOKINES/IMMUNOTHERAPY

Synergistic Effect of Temozolamide Chemotherapy And γδ T-cell-based Immunotherapy for Glioblastoma Multiforme

*Hilal Arnouk**, Lindsay Denton*, Yancey Gillespie†, Trent Spencer‡, Yun Su*, Catherine Langford†, Lawrence S. Lamb*. *Departments of *Medicine; †Surgery, University of Alabama at Birmingham, Birmingham, AL; ‡Department of Pediatrics, Emory University, Atlanta, GA*.

Background: Cellular immunity is severely depressed following standard Glioblastoma Multiforme (GBM) therapies that are based on the Stupp method. Immunotherapy presents a potential solution, and we have shown earlier that expanded/activated γδ T cells from healthy donors are cytotoxic to GBM cell lines and primary GBM explants. In this report, we investigate functional properties of expanded/activated γδ T cells prepared under therapeutic-grade conditions and their potential use in synergy with temozolamide (TMZ) to eliminate resistant GBM.

Methods: Expanded/activated γδ T cells were prepared with an 18-day culture in RPMI+HuSerum+Zoledronic Acid+IL-2 (ZOL/IL-2). GBM NKG2D ligand expression and T-cell phenotype were assessed by flow cytometry of surface antigens and intracellular cytokines. Flow-based cytotoxicity assays using GBM cell lines U251MG, U87MG, primary GBM explants, and TMZ-sensitive and TMZ-resistant D54MG determined the potency of the γδ T-cell product.

Results: The ZOL/IL-2 culture expanded γδ T cells between 200-fold to 500-fold ex vivo resulting in a product containing 65% to 80% γδ T cells. These cells were effector-memory phenotype, expressed T_H1 cytokines, tumor necrosis factor-α, and interferon-γ but not interleukin (IL)-2, IL-6, Fox-P3, IL-17, or granulocyte colony-stimulating factor. They were highly cytotoxic against U251MG, U87MG, and D54MG cell lines. Activity against $CD133^+$ progenitor cells from primary GBM explants was also seen. Although NKG2D ligands, MIC-B, ULBP-2, and ULBP-3, were expressed by GBM cell lines, blocking of these only partially reduced killing by γδ T cells. TMZ treatment of D54MG cultures resulted in the lysis of approximately 80% of cells, upregulation of MIC-B, and enrichment of $CD133^+$ cells. Expanded/activated γδ T cells completely eliminated the TMZ-resistant MIC-B+$CD133^+$ cell population and reduced the MIC-B-$CD133^+$ population over 4 hours.

Conclusions: Expanded/activated γδ T cells can be produced using therapeutic-grade materials and are highly cytotoxic to GBM and GBM progenitors. TMZ upregulates stress-associated NKG2D ligands on GBM and sensitizes them to killing by γδ T cells providing a potential approach to combine chemotherapy and immunotherapy.

A Pilot Study of Denileukin Diftitox in Combination With High-dose Interleukin-2 for Patients With Metastatic Renal Cell Carcinoma

Elizabeth A. Atchison, J. Eklund, B. Martone, L. Wang, G. MacVicar, A. Rademaker, C. Goolsby, Timothy M. Kuzel. *Feinberg School of Medicine-Northwestern University, Chicago, IL*.

Introduction: High-dose (HD) interleukin (IL)-2 is approved to treat renal cell carcinoma (RCC) but has modest response rates and high toxicity; still, IL-2 induces durable remissions in 7% to 10% of patients making it an attractive option. IL-2 is thought to work by enhancing cytotoxic T-cell activity. Unfortunately, IL-2 also stimulates T regulatory cells (Tregs) ($CD4^+CD25hi+$). In patients with RCC, Treg counts are elevated and Treg elevation has been associated with poor prognosis. Conversely, studies of immunotherapy agents have correlated favorable outcomes with a greater rebound absolute lymphocytosis (peak ALC) (Fumagalli, 2003). Denileukin Diftitox (DD) targets IL-2 receptor (CD25 component)-expressing cells. We hypothesized that sequential therapy with the 2 agents could complement each other; DD would deplete Tregs so IL-2 could more effectively stimulate the proliferation and activity of cytotoxic T cells leading to an enhanced antitumor response.

Objectives:

1. Explore whether HD IL-2 with DD is tolerable in either of 2 dosing schedules and whether therapy alters circulating levels of Tregs/enhances peak ALC
2. Identify flow cytometric and clinical predictors of response rate.

Patients and Methods:

- All patients (n = 18) received standard HD IL-2 (d 1-5, d 15-19), periodic flow cytometry (n = 14) and CBCPlease provide the full form of "CBC and MSKCC." (n = 15), and 1 dose of DD daily for 3 days (n = 18)

Groups:

First 3 patients

- Toxicity only (no flow/efficacy)
- 6 mg/kg DD between IL-2 courses (d 8-10)
  3:2 Randomization
- Toxicity and clinical efficacy (RECIST) 8–10 weeks post-treatment
- Group B (9 pts): 9 mg/kg DD before IL-2 (d-4 to d-1)
- Group C (6 pts): 9 mg/kg DD between IL-2 courses (d8-10)

Flow cytometry control: 12 patients from a study of IL-2 without DD.

Results: Of the 15 patients in groups B and C, 7 were male, mean age was 56 years, MSKCC risk group: 5 good, 5 intermediate, and 5 poor. Eight received prior systemic therapy. The median (med) number of IL-2 doses received was 22 (of 28). No unusual adverse events were noted.

- For groups B + C, the med Treg at baseline (BL) was 700/μL
- B + C med decline in Tregs was 31.4% from pre-DD to post-DD ($P = 0.013$)
- Med peak ALC change from BL was 9980/iL for B, 4470/μL for C ($P = 0.005$ for diff), and 4720/μL for control ($P = 0.035$ for diff from B)
- Med peak eosinophil change from BL was 5220/μL for B + C and 2200/μL for control ($P = 0.04$ for diff)
- Overall response rate: 4 of 13 (31%); 3 of 8 (37.5%) for B, and 1 of 5 (20%) for C (1 pt/ group too early for clinical outcome evaluation)
- Only 1 blood count parameter differed significantly between responders and nonresponders: peak eosinophilia. Med peak eosinophil change from BL was 2400/μL in responders and 8200/μL in nonresponders ($P = 0.021$ for diff)

Conclusions: DD depletes Tregs and enhances peak ALC associated with HD IL-2. Responders included 2 patients with sarcomatoid RCC and 1 with prior sunitinib therapy. Pre – IL-2 DD regimen was selected for further testing.

Global Gene Expression in Activated $CD56^{DIM}CD16^{+}$ Human NK Cells

*Neela S. Bhave**, Robin Parihar*, Nisha Shah*, Sandya Liyanarachchi*, Sri Vidya Kondadasula*, Ramana Davuluri†, William E. Carson*. **Comprehensive Cancer Center, The Ohio State University, Columbus, OH; †Bioinformatics, The Wistar Institute, Philadelphia, PA.*

Human $CD56^{dim}CD16^{+}$ natural killer (NK) cells play a critical role in the early recognition of antibody-coated targets such as foreign pathogens, allergic stimuli, and tumor cells. Traditionally, this subset of NK cells has been thought to mediate cellular cytotoxicity against antibody-coated targets, with only modest cytokine secretion capacity. Recent studies have shown an expanded role for $CD56^{dim}CD16^{+}$ NK cells in innate immunity, suggesting diverse functions for this NK cell compartment during immune responses within inflammatory tissue. NK cells also express activating receptors (FcR), which bind to the Fc region of an antibody. We report here genome-wide changes in the gene expression profiles of $CD56^{dim}CD16^{+}$ NK cells activated by interactions with the Fcγ receptor, both in the absence and presence of the proinflammatory cytokine, interleukin (IL)-12. The current study using Genechip microarrays showed that exposure of NK cells to IgG in the presence of IL-12 led to the expression of a unique set of genes that were not expressed in NK cell control conditions. Genes that were regulated > 2-fold and had a P value < 0.01 when compared with controls were considered statistically significant. Our data show that stimulation of NK cells via FcR and IL-12 significantly enhanced the secretion of inflammatory cytokines such as interferon-γ, MIP1-α, and tumor necrosis factor-α. Data analysis and comparison from 8 different biologic replicates (normal human donor NK cells) showed that in response to the IgG and IL-12 costimulation 243 genes were uniquely up-regulated and 215 genes were uniquely down-regulated in NK cells. Gene sets that were uniquely regulated included genes encoding NK cytotoxicity receptors (CD244, KLFR1, and CD300a), apoptotic proteins (eg, ASC, BNIP3L, etc), intracellular signaling molecules (ZAP-70), and cytokines (IL-3, IL-6ST, and vascular endothelial growth factor). These genes could mediate enhanced cytotoxicity and enable $CD56^{dim}CD16^{+}$ NK cells to interact with other innate and adaptive immune effectors within an inflammatory tissue. These results are the first to address the global mechanisms by which NK cells may mediate their biologic functions when encountering antibody-coated targets within inflammatory sites.

IGF-IR Peptide-based Vaccines Inhibit Tumor Growth in a Murine Model of Breast Cancer

*Denise L. Cecil**, Kyong Hwa Park†, Ekram Gad*, Doreen Higgins*, Jennifer Childs*, Mary L. Disis*. **Tumor Vaccine Group, University of Washington, Seattle, WA; †Division of Oncology/Hematology, Korea University, Seoul, South Korea.*

Insulin growth factor (IGF)-I receptor (IGF-IR) is immunogenic in breast cancer. We hypothesize that vaccination with MHCII IGF-IR – specific peptides will induce an antitumor immune response in breast cancer. IGF-IR is nearly 100% homologous between mice and humans. Neu-transgenic mice (neu-tg) have been shown to naturally overexpress IGF-IR. Therefore, we immunized neu-tg mice with IGF-IR peptides p384-398, p575-589, p951-965, and p1122-1136 to evaluate immune responses as these peptides elicited a significant interferon-γ response in peripheral blood mononuclear cells of breast cancer donors. The implanted tumor growth was inhibited by more than 70%, measured at 35 days after tumor challenge. Moreover, the IGF-IR peptide-specific tumor inhibition was dependent on $CD4^{+}$ T cells. In addition, preliminary data suggest that IGF-IR – specific immunization results in the up-regulation of tumor-associated caspase-3 expression and the antiproliferation protein PTEN, whereas overall proliferation, measured by PCNA staining, is decreased. Resistance to HER2 monoclonal antibody therapy has been associated with IGF-IR activation as well as loss of PTEN expression. Neu-tg mice do not respond completely to 7.16.4, a neu-specific monoclonal antibody that has been shown to have a therapeutic effect on these animals. We questioned whether the biologic modulation of PTEN induced by IGF-IR immunization could enhance the therapeutic efficacy of neu-specific monoclonal antibody therapy in these animals. We showed that tumor volumes in mice receiving 7.16.4 alone were reduced about 90% of those seen in control tumors, but no mice were cured. However, 3 of 4 tumors in IGF-IR-vaccinated mice receiving 7.16.4 therapy completely regressed. Thus, active immunization targeting IGF-IR may induce both immunologic and biologic effects resulting in the inhibition of breast cancer growth.

IGF-IR is a Tumor Antigen in Patients With Breast Cancer

*Denise L. Cecil**, Kyong Hwa Park†, Ekram Gad*, Doreen Higgins*, Jennifer Childs*, Mary L. Disis*. **Tumor Vaccine Group, University of Washington, Seattle, WA; †Division of Oncology/Hematology, Korea University, Seoul, South Korea.*

The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The majority of breast tumors overexpresses the IGF-I receptor (IGF-IR). Increased IGF-IR signaling in breast cancer is correlated with poor prognosis and is associated with disease that is resistant to chemotherapy, radiotherapy, and biologic therapy such as trastuzumab. Thus,

therapeutically targeting the IGF-IR signaling pathway is a promising approach to treating breast cancer. We have determined that IGF-IR is immunogenic in breast cancer. We show that patients with breast cancer have IgG antibody immunity directed against IGF-IR. As has been shown that natural immunogenic human epitopes can be predicted by high binding affinity across multiple class II alleles, we used a combined scoring system from 5 algorithms for predicting class II binding to determine Th epitopes of IGF-IR. In preliminary studies, we have identified 20 potentially immunogenic peptides, 10 in the extracellular domain, 1 in the transmembrane domain, and 9 in the intracellular domain, with 3 peptides encoding sequences essential for IGF-IR kinase function. We have observed that 95% of the peptides predicted an induced T-cell immune response as measured by interferon-γ enzyme-linked immunosorbent spot and at least 5 of the peptides can elicit an IGF-IR protein-specific T_H1 response in human peripheral blood mononuclear cells. T-cell lines generated from IGF-IR peptides show varying affinity for the recombinant protein. IGF-IR is highly homologous with human insulin receptor. We show that at least 2 peptides can generate T cells that secrete both interferon-γ and interleukin-10, suggesting that some IGF-IR peptides might induce an immunosuppressive phenotype. These data suggest IGF-IR may be a target for active immunization.

Differential Modulation of Hepatic and Splenic Dendritic Cell Number and Functions With Systemic Interleukin-12 Therapy

Tim Chan, Timothy C. Back, Jeffrey J. Subleski, Jonathan M. Weiss, John R. Ortaldo, Robert H. Wiltrout. *Laboratory of Experimental Immunology, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD.*

The liver is an immunologically unique organ that contains tolerogenic or immunosuppressive hepatic dendritic cells (DCs). The regulation of hepatic DCs within this microenvironment may be a contributing factor for the development of hepatic tumors and metastasis. Immunotherapeutic strategies, such as the administration of systemic interleukin (IL)-12 therapy, lead to the activation of natural killer and T cells that improves the outcome for treating established tumors. However, the effect of IL-12 – based systemic treatments on DC populations in the spleen and liver microenvironments and their contributing roles in the antitumor response has not been fully determined. In this study, we show that daily systemic IL-12 administration results in a marked 2-fold to 3-fold increase in splenic and hepatic DC populations, as defined by CD11c and major histocompatibility complex class II expression, in both naive mice and mice bearing a highly metastatic murine renal cell carcinoma (RENCA). The increased number of conventional DC (cDCs) and plasmacytoid DC (pDCs) was equivalent in the spleen, whereas in the liver, there was a greater increase in cDCs whereas pDCs remain unchanged. Enhanced expression of costimulatory molecules (CD80, CD86) was observed from systemic IL-12-treated splenic and hepatic DCs, with the latter showing a substantial increase in costimulatory molecule expression compared with vehicle control-treated mice. Interestingly, hepatic DCs from IL-12-treated mice showed greater T-cell proliferation in both allogeneic mixed lymphocyte reaction and T-cell receptor-specific T proliferation assays compared with hepatic DCs from vehicle control-treated mice. However, no significant differences in T-cell proliferation were observed with splenic DCs. Furthermore, systemic administration of IL-12 further primed splenic and hepatic DCs to produce higher levels of IL-12 in combination with various TLR agonists when cultured ex vivo. The combined differential regulation of hepatic DCs with systemic IL-12 therapy suggests the potential for IL-12 to overcome the inherent suppressive components of the hepatic environment. Potentially, this study may impact the design of IL-12 – based immunotherapeutic strategies to optimally activate the innate and adaptive immune response for primary and metastatic tumors located in the liver.

Cimavax EGF, Vaccine for Nonsmall Cell Lung Cancer Treatment: Interim Analysis of a Phase III Clinical Trial

*Tania Crombet**, E. Neninger†, B. Garcia*, Gisela Gonzalez*, Agustin Lage*. **Center of Molecular Immunology, Center of Molecular Immunology;* †*Hermanos Ameijeiras Hospital, Havana, Cuba.*

Lung cancer is the leading cause of cancer-related mortality. Nonsmall cell lung cancer (NSCLC) is the most common lung cancer, characterized by being a heterogeneous aggregate of histologies. The epidermal growth factor (EGF) and its receptor (EGFR) is a high-specificity molecular complex whose interaction unchains proliferative signals essential for the development of many types of tumors. As EGFR is expressed in most cases of NSCLC, this receptor is considered as an attractive target for novel therapies.

Our approach is an active immunotherapy against the ligand, EGF. CIMAvax EGF is a vaccine that consists of human recombinant EGF conjugated to the P64k carrier protein and with Montanide ISA51 VG as adjuvant. Vaccination with CIMAvax EGF induces an immune response as antibodies that specifically recognize the EGF and inhibit its binding with the EGFR, avoiding the proliferation mechanisms derived from this binding. The clinical experience with CIMAvax EGF began in 1995, and from then on, more than 800 advanced NSCLC patients were vaccinated with proven safety. Results from earlier clinical trials showed that vaccination induces anti-EGF antibodies and decreases EGF sera concentrations. There was a significant direct correlation between antibody titers and patient survival and a significant inverse correlation between sera EGF concentrations and patient survival. Here we report the results from an interim report of a phase III trial, designed to evaluate the efficacy of CIMAvax EGF in advanced NSCLC patients who respond to first-line chemotherapy. Before randomization, the patients were stratified according to age. This analysis encompasses the stratum of patients younger than 60 years observed in phase II. Even when still not significant, evaluable patients survive more than controls in this age stratum (vaccinated: mean 26.9 mo, median 22.8 mo vs. controls mean 16.2 mo, median 17.3 mo), showing a delayed separation in the survival curves; 1-year survival 73.4% vaccinated versus 61.7% controls, 18-month survival 52.5 months versus 39.6 months, 2-year survival 45.9 months versus 29.7 months. Sixty-two percent of the vaccinated patients were classified as good antibody responders (GAR) (titers higher than 1:4000 sera dilution) and 28% were classified as sGAR (titers higher than 1:64,000). There is a direct correlation between antibody titers and survival; GAR patients survived significantly more than poor antibody responders and sGAR patients survived significantly more than GAR. Vaccination was shown to be immunogenic and safe and the correlation between survival and immunogenicity was confirmed.

Identification and Characterization of Interleukin-4 Receptor α Chain in Human Anaplastic Thyroid Carcinoma and Targeting of IL-4 Receptor for Therapy

Ritika Dogra, Bharat H. Joshi, Raj K. Puri. *Tumor Vaccine and Biotechnology Branch Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research FDA, Bethesda, MD.*

Earlier, we have reported that receptors for interleukin-4 receptor (IL-4R) are overexpressed in thyroid cancer samples in situ. In addition, we have shown that overexpression of IL-4R sensitizes tumors to a receptor-directed therapeutic agent consisting of circularly permutated IL-4 (cpIL-4) and a truncated Pseudomonas exotoxin (cpIL-4PE). cpIL4-PE is being tested in the clinic as a therapy for glioblastoma multiforme. As only a few therapeutic options are available for anaplastic thyroid carcinoma (ATC), here we have examined whether (1) anaplastic thyroid cancer cell lines express IL-4R and (2) cpIL4-PE is active in mediating anticancer effects in vitro and in vivo. Two of the 3 anaplastic thyroid cancer cell lines that were tested, expressed high levels of both IL-4R mRNA and protein. In addition, these cells also expressed

IL-13Rα1, but not IL-2γC, indicating that ATC cells express type 2 IL-4R. In a protein synthesis inhibition assay using cpIL4-PE, we found that anaplastic thyroid cancer cell lines are highly sensitive to cpIL4-PE (Ic50 < 1 ng/mL). These results were confirmed by clonogenic assays. We also developed tumors in athymic immunodeficient mice by implanting 5×10^6 anaplastic thyroid cancer cells subcutaneously and treating these animals with a 100 μg/kg dose of cpIL4-PE on 3 alternate days. Our initial results indicate that 4 of 6 mice showed significant regression of their established tumor and the other 2 showed a partial response to the treatment. Additional studies are ongoing to determine the best route and schedule of cpIL4-PE administration. These results indicate that human ATC expresses high levels of IL-4Rα, which can be targeted by cpIL4-PE.

Tumor Antigen NY-ESO-1-Derived CD8$^+$ T-cell Epitopes are Presented Differently by DCs and Melanoma Cells

Weisan Chen*, *Lisa Ebert**, Neil Robson*, Rona Zhao*, Nicole A. Mifsud*, Tristan McAlpine*, Kun Xiao*, Heather Jackson*, Nektaria Dimopoulos*, Philippe Guillaume†, Sarah E. Russell*, Emmanuel Scotet‡, Immanuel F. Luescher†, Ian D. Davis*, Eugene Maraskovsky§, Jonathan S. Cebon*. *Ludwig Institute for Cancer Research; §CSL, Pty Ltd, Melbourne, VIC, Australia; †Ludwig Institute for Cancer Research, Lausanne, Switzerland; ‡Institut de Biologie, Nantes, France.

We performed a placebo-controlled phase I clinical trial using recombinant NY-ESO-1 (hereafter ESO) protein formulated with an ISCOMATRIXTM adjuvant. High-titer antibody, strong DTH reactions, and broad CD8$^+$ and CD4$^+$ T-cell responses were observed. We have subsequently conducted detailed antigen processing and presentation experiments for a few ESO-specific CD8$^+$ T-cell epitopes using either tumor cells or antigen-loaded MoDCs. We show that different epitopes from the same ESO antigen are direct-presented or cross-presented differently. Although ESO157-165 is presented efficiently from both pathways and from MoDCs loaded with various forms of full-length ESO, ESO88-96 is not presented by the tumor cells, which naturally presented ESO157-165. However, this epitope is highly efficiently cross-presented by DCs loaded with soluble NY-ESO-1. Another epitope, ESO60-72, however, is presented endogenously by tumor cells or by DCs that are loaded with the ESO protein formulated with the ISCOMATRIXTM adjuvant, and such a presentation is enhanced by inhibiting the TPP-II antigen processing pathway. Moreover, although this response is readily vaccinated using NY-ESO-1-ISCOMATRIXTM, it is rarely detectable from melanoma patients with spontaneous immunity to ESO. Taken together, these results imply that due to the antigen presentation discrepancy between DCs and tumor cells, most likely as a result of the expression of immunoproteasome versus house-keeping proteasome, some CD8$^+$ T-cell responses are efficiently primed in vivo but maybe clinically irrelevant due to a lack of direct presentation by tumor cells; In contrast, tumor-specific T cells may remain naive, in a "sleeping" state, due to a lack of priming by DC although the related antigen epitope is presented abundantly in tumor cells. Studying the antigen processing properties of these immunodominant T-cell epitopes will help us in better understanding the vaccine design.

High-Dose Interleukin-2 Followed by Temozolomide Results in Response Rates of High Quality in Metastatic Melanoma

*Salman Fateh**, Todd Schell†, Rebecca Gingrich*, Rogerio Neves‡, Joseph Drabick*. *Departments of *Hematology and Oncology; †Microbiology and Immunology; ‡Plastic Surgery, Penn State Hershey Medical Center, Hershey, PA.*

Metastatic melanoma is a disease with a very poor prognosis typified by a median survival of less than 1 year. High-dose interleukin-2 (HD IL-2) and temozolomide (TMZ) are both approved treatments by the Food and Drug Administration for this malignancy but the response rates or these as single agents are low. HD IL-2 is the only therapy to date that has been shown to induce durable responses but only in a small percentage of patients. The combination of TMZ followed by HD IL-2 has been studied but did not improve responses over what had been observed for HD IL-2 alone. In our clinical practice, we noted dramatic responses to TMZ when given immediately to 4 sequentially treated patients who had just completed a full course of HD IL-2 and had either failed to respond or progressed. Two patients had complete or near-complete responses (CRs) and 2 had very good partial responses; the CR in one has been durable to date. These responses were much better than what is typically observed for the single agent, TMZ. TMZ is an oral atypical alkylating agent that, in addition to having cytotoxic activity against melanoma, has also been shown to decrease T regulatory populations of lymphocytes. We review these 4 cases and hypothesize that TMZ may be synergic with HD IL-2 in a sequence-specific manner by allowing the immune activation induced by the HD IL-2 to proceed without the natural brakes applied by the T regulatory population of cells whose major function is to inhibit an exuberant immune response. This postulated mechanism would result in the sequence-specific activity noted in our patients. A prospective phase II trial to test this hypothesis has been initiated.

Identification of Interleukin-13 Receptor α2 Chain as a Biomarker for Tumor Invasion and Metastasis Using a Mouse Model of Human Pancreatic Cancer

*Toshio Fujisawa**, Bharat H. Joshi*, Atsushi Nakajima†, Raj K. Puri*. **Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD; †Gastroenterology Division, Yokohama City University Graduate School of Medicine, Yokohama, Japan.*

Earlier, we have shown that the interleukin (IL)-13 Receptor α2 chain (IL-13Rα2) is overexpressed in a variety of human solid cancers including pancreatic cancer. Here, we have investigated its significance in cancer invasion and metastasis in vivo. Two pancreatic cancer cell lines, together with IL-13Rα2-positive (HS766 T) and IL-13Rα2-negative (HPAF-II) cells were studied for evaluating the role of IL-13Rα2 in invasion and metastasis. IL-13 treatment enhanced cell invasion in IL-13Rα2-positive cancer cell lines but not in IL-13Rα2-negative cell lines. Gene transfer of IL-13Rα2 into a negative cell line (HPAF-II) enhanced invasion, whereas its silencing in a positive cell line (HS766 T) using RNAi down-modulated invasion in a matrigel invasion assay. We developed an athymic nude mouse model of human pancreatic cancer for tumor metastasis by implanting orthotopic tumors. In this model, IL-13Rα2-positive tumors metastasized to lymph nodes, liver, and peritoneum at a significantly higher rate compared with IL-13Rα2-negative tumors. IL-13Rα2 expression in metastatic lesions was increased compared with primary tumors, and mice with IL-13Rα2-positive cancer displayed cachexia and had a poor prognosis. Our results show for the first time that IL-13Rα2 may have a critical role in cancer progression and can serve as a prognostic biomarker of pancreatic cancer invasion and metastasis.

Role of Interferon-γ – Indoleamine 2,3 Dioxygenase Axis in the Regulation of Interleukin-12 – mediated Antitumor Immunity

Tao Gu, Rachael B. Turner, Mehmet O. Kilinc, Nejat K. Egilmez. *Microbiology and Immunology, University at Buffalo, Buffalo, NY.*

Intratumoral interleukin (IL)-12 and granulocyte macrophage-colony-stimulating factor induce tumor regression via the restoration of tumor-resident CD8$^+$ T-effector-memory cell cytotoxicity and subsequent repriming of a secondary CD8$^+$ T-effector cell response in tumor-draining lymph nodes (TDLN). However, posttherapy T-effector activity is limited to a 7-day to 10-day window and is accompanied with CD4$^+$ CD25$^+$ Foxp3+ T-suppressor cell expansion. Molecular and cellular changes in posttherapy tumor microenvironment and TDLN were monitored

to elucidate the mechanism of counter-regulation. Real-time polymerase chain reaction analysis revealed a 5-fold enhancement of indoleamine 2,3 dioxygenase (IDO) expression both in the tumor and the TDLN 1 day after treatment. IDO induction required interferon-γ and persisted for up to 7 days. Administration of the IDO inhibitor, D-1-methyl tryptophan, concurrent with treatment resulted in a dramatic enhancement of tumor regression. Enhanced efficacy was associated with a diminished $CD4^+$ Foxp3+ T-suppressor cell rebound, revealing a link between IDO activity and posttherapy T-suppressor cell expansion. Further analysis established that abrogation of the regulatory counter-response resulted in a 10-fold increase in the intratumoral $CD8^+$ T cell to $CD4^+$ Foxp3+ T-cell ratio. The ratio of proliferating $CD8^+$ T-effector to $CD4^+$ Foxp3+ T-suppressor cells was prognostic for the efficacy of tumor suppression in individual mice. Interferon-γ–dependent IDO induction and T-suppressor cell expansion were primarily driven by IL-12. These findings show a critical role for IDO in the regulation of IL-12–mediated antitumor immune responses.

In Vivo Overexpression of Interleukin-4 Receptor α in a Mouse Model of Human Bladder Carcinoma Sensitizes Tumors to Recombinant Chimeric Immunotoxin Consisting of Interleukin-4 and Pseudomonas Exotoxin

Bharat H. Joshi, Pamela Leland, Raj K. Puri. *Tumor Vaccines and Biotechnology Branch, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, MD.*

Interleukin-4 receptor α (IL-4Rα), a primary IL-4 binding subunit of IL-4R complex overexpressed in many different types of human cancers, is an excellent target for targeted cancer therapies. A chimeric protein consisting of circularly permutated IL-4 and a truncated form of *Pseudomonas* exotoxin [IL-4(38-37)-PE38K-DEL], termed cpIL-4PE, mediates between the remarkable antitumor effects in animal models of human cancer. cpIL-PE binds to IL-4R–positive tumor cells with high specificity and then it is internalized and processed in the intracellular compartment and blocks protein synthesis resulting incell death. We have reported that human bladder carcinoma cell lines overexpress type 2 IL-4Rα and that cpIL-4PE is high-to-moderately cytotoxic to these cell lines in vitro. In this study, we have developed a subcutaneous mouse model of human bladder cancer by implanting 5×10^6 UM-UC-3 bladder cancer cells in athymic nude female mice and the antitumor effect of cpIL-4-PE was tested. Intratumoral treatment with cpIL-4PE (100 μg/kg) eliminated small tumors (16-20 mm^2) in 6 of 12 (50%) mice, partially regressed tumors in 33% (4/12) (60% tumor shrinkage) and no response in 2/12 (17%) mice. In contrast, 67% (8/12) mice with large tumors partially responded by reducing the tumor growth to 40% of controls. We are currently testing the efficacy of cpIL-4PE by the intraperitoneal route of administration. Thus, human bladder carcinoma cells overexpress type II IL-4R in situ and these receptors may serve as a novel target for bladder cancer therapy.

Clinical Activity Following the Administration of the MAGE-A3 Antigen-specific Cancer Immunotherapeutic in Both the Melanoma and NSCLC is Associated With Similar Predictive Biomarkers Present Prior Treatment

*F. Lehmann**, F. Ulloa-Montoya*, O. Gruselle*, B. Dizier*, Jamila Louahed*, J. Vansteenkiste†, A. Spatz‡, W. Kruit§, V. Brichard*. **GlaxoSmithKline Biologicals, Rixensart;* †*University Hospital Gasthuisberg, Leuven, Belgium;* ‡*Jewish General Hospital, Montreal, QC, Canada;* §*Erasmus Medical Center, Rotterdam, Netherlands.*

Background: Antigen-specific cancer immunotherapeutics (ASCI) are made of a tumor-specific antigen, delivered as a recombinant protein combined with a GSK proprietary immunostimulant designed to enhance the immune response to the injected antigen. The most advanced ASCI under development targets MAGE-A3, a tumor-specific antigen not expressed on normal cells but shared by a variety of tumor types, specifically on 65% of metastatic melanomas and 35% of nonsmall cell lung cancer (NSCLC).

Methods and Results: A randomized open-label phase II study (EORTC 16032-18031) was conducted to evaluate MAGE-A3 recombinant protein combined with AS02B and AS15 as first-line metastatic treatment in MAGE-A3(+) cutaneous melanoma patients. Gene expression profiling (GS) by microarrays was performed on the tumor that had taken prior treatment to identify biomarkers predictive of the observed clinical activity of the MAGE-A3 ASCI. Overall survival was improved significantly in the population of patients whose tumor presented the predictive signature: 16.2 months in the GS(−), 28 months in the GS(+) population. Although the gene signature was randomly distributed between both study arms, a more important effect on overall survival was observed in the AS15 arm. Most of the identified genes are immune-related, defining a particular biologic context present in the tumor environment before immunization. Whether the predictive value of the melanoma signature was applicable to NSCLC was evaluated on samples from a double-blind placebo-controlled phase II study of MAGE-A3 ASCI in NSCLC. The relative reduction in the risk of recurrence upon MAGE A3 ASCI treatment is increased by about 2-fold in the patients with the predictive gene signature as compared with the overall population: from 25% relative improvement in the overall population to 46% in patients whose tumor presents the predictive gene signature. No significant difference in DFS was observed between GS(+) and GS(−) patients in the placebo group, suggesting this signature has a real predictive value and no prognostic value in this patient population.

Conclusions: In conclusion, biomarkers predictive of clinical activity of the MAGE-A3 ASCI treatment were identified in melanoma and confirmed in NSCLC. The initiation of phase III studies in NSCLC (MAGRIT) and melanoma (DERMA) is a unique opportunity to validate this gene expression set prospectively and select ultimately the patients that are the most likely to benefit from the MAGE-A3 ASCI therapy.

Expression of the Interferon-λ Gene With Adenoviruses-Induced Cytotoxic Effects on Esophageal Carcinoma

*Quanhai Li**, Kiyoko Kawamura*, Shinya Okamoto*†, Hiroshi Kobayashi†, Yuki Takei*‡, Naoto Yamaguchi‡, Fumi Iwata§, Muneo Numasaki§, Hideaki Shimada‖, Masatoshi Tagawa*. **Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute;* †*Departments of Biochemistry;* ‡*Molecular Cell Biology, Graduate School of Pharmaceutical Sciences, Chiba University;* ‖*Division of Gastroenterological Surgery, Chiba Cancer Center, Chiba, Japan;* §*Department of Nutritional Physiology, Faculty of Pharmaceutical Science, Josai University, Sakado, Japan.*

Novel cytokines, interleukin-28A (IL-28A), IL-28B, and IL-29 have been identified to belong to a novel interferon (IFN) family, IFN-λs. All the 3 cytokines have similar functions and IFN-λs are classified as a type III IFN. However, detailed biologic properties of IFN-λs, in particular regarding the growth inhibition activity, remain uncharacterized. We found that human esophageal carcinoma cells expressed the IFN-λs receptor complex and that recombinant IFN-lλs upregulated the expression level of the class I molecules of the major histocompatibility complexes and induced the gene expression of MxA and 2′,5′-oligoadenylate synthetase, which are involved in antiviral protection, in all the cells tested. We also found that IFN-λs inhibited proliferation of esophageal carcinoma cells through multiple mechanisms including G1 cell cycle arrest or apoptosis, which was accompanied by upregulated p21 expression or increased Annexin-V–positive cells, respectively. Interestingly, all the esophageal carcinoma cells expressed the IFN-λ receptor complexes and IFN-λ upregulated major histocompatibility complex class I expression but inhibited proliferation was not observed in all the cells tested. We then prepared adenoviruses (Ad) bearing the IL-28A gene and found that the Ad inhibited the growth of IFN-λ–sensitive carcinoma

cells. Human fibroblasts were insensitive to IFN-λ as they did not express the IFN-λ receptor complex. Transduction of the fibroblasts with the Ad produced IL-28A and the growth was not inhibited at all in contrast with sensitive esophageal cells. A mixed cell culture of fibroblasts secreting IL-28A and esophageal carcinoma cells suppressed tumor growth but not the growth of fibroblasts. These data suggest that implantation of fibroblasts transduced with the Ad-bearing IFN-λs into tumors achieved antitumor effects, which was evidenced by our experimental system. IFN-λs also produced additional antitumor effects in combination with anticancer agents. These data collectively show that IFN-λ is a potential anticancer drug for esophageal carcinoma and the Ad-producing IFN-λs could be useful in combination with chemotherapeutic agents.

Polysaccharide Krestin Stimulates Interleukin-12 Production in Dendritic Cells and the Development of Antigen-specific T Cells via TLR2 Activation

Hailing Lu, Yi Yang, Ekram Gad, Mary L. Disis. *Tumor Vaccine Group, University of Washington, Seattle, WA.*

Polysaccharide krestin (PSK) is a mushroom extract that has long been used in Asia as an anticancer and immunostimulatory drug, but the mechanism of action is unclear. Using ova-transgenic mice, we found that PSK can activate dendritic cells (DCs) and augment an antigen-specific T-cell response. We then studied the immunomodulatory effect of PSK in neu transgenic mice, a model of human HER2-positive breast cancer that mimics the self-tolerance and dampened immunity in cancer patients. In vitro PSK stimulates splenocyte proliferation and activates bone marrow-derived DC to secrete interleukin-12. Oral administration of PSK significantly inhibits the growth of both implanted and spontaneous breast tumors in these mice. PSK treatment results in increased T-cell infiltration into the tumors of these animals and creates a T_H1 shift in the tumor microenvironment. Furthermore, PSK activates DCs in both tumor-draining lymph node and mesenteric lymph node. To evaluate whether PSK was activating toll-like receptors (TLRs) on DCs, we used HEK293 cells transfected with different TLRs and incubated them with PSK (0.5 to 1500 μg/mL, 16 h). PSK-activated nuclear factor-κB in HEK cells transfected with TLR2 in a dose-dependent manner, but had no effect on HEK transfected with other TLRs (3, 4, 5, 7, 8, or 9). To further investigate the role of TLR2 in mediating the immunostimulatory effect of PSK, we used splenocytes from TLR2 −/− mice and wild-type mice and treated them side-by-side with PSK (25 to 400 μg/mL, 72 h). PSK stimulates the secretion of tumor necrosis factor-á in splenocytes from wild-type mice dose-dependently but not in splenocytes from TLR2 −/− mice. PSK also failed to activate DCs from TLR2 −/− mice. These results suggest that the immunostimulatory effects of PSK on DCs and T cells are mediated via TLR2.

Interleukin-15 Administration to Rhesus Macaques Expands Natural Killer and Memory T Cells and Induces Long-lasting Changes in T-cell Homeostasis

*Enrico Lugli**, Carolyn K. Goldman†, Mario Roederer*, Thomas A. Waldmann†. **VRC; †CCR, NIH, Bethesda, MD.*

Several human diseases display disorders of the immune system; for example, due to virus infection or to the indirect effect of cancer cells. Thus, boosting immunity in these patients may be beneficial for the development of a successful treatment. The γ-chain family cytokine interleukin (IL)-15 has this potential as it has been shown to efficiently expand natural killer (NK) cells and memory T cells in vivo. For a better understanding of the dynamics of these populations in vivo, we administered IL-15 intravenously to rhesus macaques at 10, 20, and 50 μg/kg for 12 days and monitored several lymphocyte populations in the peripheral blood (PB) until 48 days after the first injection [postinjection (p.i.)], and in the lymph nodes, spleen, bone marrow, and jejunum at day 13 and day 48 p.i. By 10–14 color flow cytometry, we analyzed T-cell differentiation (by CD45RA, CCR7, CD95, and CD28), activation (by HLA-DR, CCR5, CD25, and CD38), proliferation (by Ki-67), and NK-cell subsets (by CD56 and CD16).

IL-15 provoked transient lymphopenia in the PB at day 2 p.i., which preceded a dose-dependent NK and T-cell expansion. This occurred at day 8, was maintained until day 15, and rapidly returned to baseline after IL-15 was stopped. IL-15 expanded $CD56^-CD16^+$ NK cells and memory T cells and, preferentially, effector (T_{EM}) more than central (T_{CM}), transitional (T_{TM}), or terminal effector (T_{EMRA}) memory cells. By contrast, naive T (T_N) cells were minimally increased in the PB. T cells were activated and proliferating, that is, $CD25^+$, $CD38^+$, and Ki-67+. IL-15 also induced its own receptor, that is, the CD122 and CD132 molecules. Memory, but not T_N, in tissues at day 13 were activated and were Ki-67+. The hierarchy of stimulation was the same as in the PB, that is, $CD8^+ > CD4^+$ and $T_{EM} > T_{CM} > > T_N$ cells. The CD4/CD8 ratio was inverted in most tissues but this was not due to direct expansion by the cytokine in situ, as the proportion of T_N did not change with treatment. Instead, it was likely due to preferential retention of $CD8^+$ cells subsequent to lymphopenia at day 2. This was associated with a body-wide differentiation of T_{CM} cells into T_{TM} or T_{EM}. Conversely, NK-cell expansion occurred only in the spleen and involved $CD56^-CD16^+$ cells. These latter changes were not observed at day 48 p.i. nor did the redistribution of expanded cells to other tissues occurr. By contrast, we found that the CD4/CD8 ratio was still decreased in IL-15 − treated animals versus sham animals. In conclusion, we show that (i) IL-15 expands NK cells and memory T cells; (ii) stimulation occurs in secondary lymphoid tissues and the bone marrow for T cells and in the spleen for NK cells; and (iii) the balance of naive/memory T cell and CD4/CD8 T cells is differentially regulated after acute stimulation by the cytokine.

PI3K Targeting by the β-GBP Cytokine in the Biological Therapy of Cancer

Livio Mallucci, Valerie Wells. *School of Biomedical and Health Sciences, King's College London, London, United Kingdom.*

In previous studies we have shown that class 1A and class 1B phosphoinositide 3-kinases (PI3Ks) are under the control of the β-GBP molecule (monomeric β-galactoside binding protein), an antiproliferative cytokine produced by $CD4^+$ and $CD8^+$-activated T cells.[1] Using the recombinant form of this cytokine (Hu-r-β-GBP), we have shown that β-GBP induces functional inhibition of the p110 catalytic subunit of PI3Ks in a mode similar to the pharmacologic inhibitors, wortmannin and LY294002.[2] Down-regulation of PI3K activity by the β-GBP cytokine leads to loss of Ras-GTP loading[2] and loss of akt gene expression.[3] In cancer cells, but not in normal cells, these conditions lead to apoptotic death.

Currently, we find that in colon cancer cells, chosen for high mutational complexity, inhibition of PI3K activity by minimal doses of β-GBP (1 to 4 nM) is followed by 2 sets of events, which initiate with cytoskeletal rearrangement. One is characterized by the overexpression of cyclin E, inhibition of DNA synthesis, and Chk2 activation, a condition for the activation of intrinsic apoptosis. The other relates to macromolecular changes in the plane of the membrane characterized by CD95/Fas clustering, a condition for the activation of extrinsic apoptosis. Considerations of the effect of β-GBP on PI3K activity and downstream signaling on different effectors in diverse cell systems suggest different roles for different PI3K isoforms.

The importance of PI3K activity in leading to and maintaining cancerogenesis has prompted the development of inhibitors aimed at the targeting of PI3K for therapeutic intervention. Our studies indicate that this can be achieved by the use of the β-GBP cytokine whose physiologic nature allows selective signaling adaptations according to cancer cell type, a property outside the ability of drugs, further to not carrying therapeutic disadvantages.

References:
1. Blaser C, et al. *Eur J Immunol*. 1998;28:2311–2319.
2. Wells V, Downward J, Mallucci L. *Oncogene*. 2007;26: 7709–7714.
3. Wells V, Mallucci L. *Breast Cancer Res*. 2009;11:R2 (doi;10.11.86/bcr2217).

Effects of Continuous Immunization With MVA-BN®-HER2: Tolerability and Immune Responses Toward Transgene and Vector

Stefanie Mandl, Ryan Rountree, Katie Dalpozzo, Lisa Do, Huiqing Hu, Deepa Sunkara, Reiner Laus, Alain Delcayre. *BN ImmunoTherapeutics, Mountain View, CA*.

BN-ImmunoTherapeutics (BNIT) is currently developing the recombinant, replication-selective vaccinia virus, MVA-BN®-HER2, as a candidate immunotherapy product for the treatment of breast cancers. MVA-BN®-HER2 is a recombinant MVA-BN® vector that encodes a modified form of the HER-2 protein, referred to as HER2. This product has been tested in preclinical studies and in 2 phase I clinical trials (BNIT-BR-001 and BNIT-BR-002) and has proven to be safe and immunogenic in both mice and humans. Earlier preclinical studies conducted at BNIT have shown that the treatment of mice with MVA-BN®-HER2 can elicit HER-2 – specific immune responses and inhibit the growth of HER-2 – positive tumors in both prophylactic and therapeutic settings. In these studies, the treatment schedule generally consisted of 3 administrations. In humans, HER-2 is expressed on selected normal tissues as well as being described as a tumor-associated antigen. As a result, humans are peripherally tolerant to HER-2 and repeated treatment may be required for maximal antitumor efficacy in cancer patients. Thus, for the impending phase I/II trial, BNIT proposes to increase the number of treatments from 3 to 6. The studies described here were carried out to evaluate the effects of continuous treatment with MVA-BN®-HER2 with respect to (a) vector tolerability and (b) immune responses toward transgenes and vectors in mice.

The preclinical studies described here assessed the tolerability of 8 consecutive immunizations with a high dose of MVA-BN®-HER2 (5E7 TCID50) in female BALB/c mice. In addition, we investigated the effect of multiple immunizations on MVA-specific and HER-2 – specific immune responses in BALB/c and C57BL6 mice. Anti-HER-2 and anti-MVA antibody and T-cell responses were evaluated by enzyme-linked immunosorbent assay and enzyme-linked immunosorbent spot, respectively. Repeated immunizations (up to 8) with MVA-BN®-HER2 product were well tolerated and no systemic toxicity in clinical pathology, gross pathology, organ weight, or histopathology parameters was observed in female mice treated with MVA-BN®-HER2. Transient, treatment-related subacute inflammation was found only locally at the injection sites of MVA-BN®-HER2 – treated animals. Each of the first 3 treatments with MVA-BN®-HER2 led to increasingly higher antibody responses and improved longevity of antibody titers after the cessation of treatment. Antibody responses in mice treated 3 or more times were similar in both magnitude and longevity. Increasing the number of doses from 3 to 7 did not further increase the cellular response to the vector. Other aspects of the induced immune responses are currently under investigation.

Both Innate and Adaptive Effector Cells Contribute to the Antitumor Activity of MVA-BN®-HER2, a Vaccine Candidate in Development for the Treatment of HER-2 – positive Breast Cancer

Stefanie J. Mandl, Ryan Rountree, Katie Dalpozzo, Lisa Do, Deepa Sunkara, Huiqing Hu, Reiner Laus, Alain Delcayre. *BN ImmunoTherapeutics, Mountain View, CA*.

MVA-BN®-HER2 is a MVA-BN® – derived, replication-deficient vector designed for the treatment of HER-2 – positive breast cancer. MVA-BN®-HER2 encodes a modified form of the HER-2 protein, referred to as HER2. We have shown earlier that MVA-BN®-HER2 is a potent immunogen, T_H1 adjuvant, which induces antigen-specific humoral and cellular responses in mice and humans and shows therapeutic activity in several mouse tumor models. Here, we present further insight into the mechanism of action of MVA-BN®-HER2 – induced antitumor activity in a murine experimental lung metastasis (ELM) model (CT26-HER-2). In the CT26-HER2 ELM model, the tumor burden can be easily determined by comparing the lung weight of tumor-bearing mice with that of naive mice, and dissociation of lung tissue allows for the phenotypic analysis of tumor-infiltrating cells. The role of natural killer (NK)cells, $CD8^+$ cells, and $CD4^+$ cells was additionally evaluated by in vivo depletion using antibodies specific to the respective cell type.

Early treatment (day 1) of CT-26-HER2 ELM with either the MVA-BN® vector alone or MVA-BN®-HER2 inhibited tumor growth. In vivo NK cell depletion reduced the antitumor activity of MVA-BN® but had no effect on the activity of MVA-BN®-HER2. These data suggest that the antitumor activity of MVA-BN®-HER2 includes the induction of innate immunity provided by the characteristics of the parental MVA-BN® vector. When therapeutic intervention was delayed (treatment day 4), only treatment with MVA-BN®-HER2, but not MVA-BN, led to strong antitumor activity. Phenotypic analysis revealed the infiltration of the MVA-BN®-HER2 – treated lungs by activated, HER-2 – specific $CD8^+$ $CD11c^+$ T cells. No significant changes were detected in the spleens of treated animals. Furthermore, only treatment with MVA-BN®-HER2 resulted in a reduction in tumor-infiltrating T regulatory cells. Depletion of CD8+ T cells reduced antitumor efficacy, confirming the importance of $CD8^+$ T cells as effector cells. Both the early and late activity of these vectors required live virus. We also compared treatment of MVA-BN®-HER2 with treatment with HER2 protein formulated in complete Freud's adjuvant (CFA). No significant tumor infiltration with $CD8^+$ T cells was observed in CFA + HER2-treated animals. In contrast to MVA-BN®-HER2, which promotes T_H1-biased antibody responses, administration of CFA + HER2 protein induced a strong Th2-biased antibody response. However, this was not sufficient for antitumor activity. Taken together, our data show that MVA-BN®-HER2 controls tumor growth through a combination of innate and adaptive immune responses.

High Immunogenic Potential of P53 MRNA-transfected Dendritic Cells in Patients With Primary Breast Cancer

*Özcan Met**, Eva Balslev†, Henrik Flyger‡, Inge Marie Svane*. **Center for Cancer ImmuneTherapy, Department of Hematology; and Department of Oncology; Departments of † Pathology; ‡Breast Surgery, University Hospital Herlev, Copenhagen, Denmark*.

As preexistent immunity might be a reflection of an emerging anticancer response, the demonstration of spontaneous T-cell responses against tumor-associated antigens (TAAs) in cancer patients may be beneficial before the clinical development of dendritic cell (DC)-based cancer vaccines, because it will help identify likely responders to TAAs among patients who qualify and may benefit from this form of immune therapy. This study aimed to determine preexistent T-cell reactivity against the tumor suppressor protein p53 in breast cancer patients at the timeof primary diagnosis. After a short-term stimulation with autologous wt p53 mRNA-transfected DCs, interferon (IFN)-γ enzyme-linked immunosorbent spot analysis revealed p53-reactive T cells in the peripheral blood of more than 40% (15 of 36) of the tested patients. Both $CD4^+$ and $CD8^+$ p53-specific T cells secreted IFN-γ after stimulation with p53-transfected DCs. Interestingly, more than 72% (13 of 18) of patients with high p53 ($p53^{high}$) expression in tumors were able to mount a p53-specific IFN-γ T-cell response, whereas p53-reactive T cells were detected in only 10% (1 of 10) of healthy donors and 11% (2 of 18) of patients with low or absent p53 ($p53^{low}$) expression in tumors. Furthermore, significantly higher secretion of interleukin-2 was detected in peripheral blood mononuclear cells after stimulation with p53-transfected DCs from

patients with $p53^{high}$ tumor expression compared with patients with $p53^{low}$ tumor expression, whereas secretion of interleukin-10 was predominant in the latter group. The high frequency of spontaneous wt p53-reactive T cells detected in the peripheral blood of primary breast cancer patients with the accumulation of p53 in tumor provides a rationale for considering DCs transfected with mRNA encoding wt p53 for clinical investigation in these patients.

Interleukin-24 Regulation of Homeostasis in the Skin Microenvironment During Inflammation

Nancy J. Poindexter, Elizabeth A. Grimm. *Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX.*

Interleukin (IL)-24 is a member of the IL-10 family of cytokines that is normally expressed by immune cells while its target seems to be cells in the skin. Our experiments addressing the role of IL-24 in human skin have shown that IL-24 is upregulated during the inflammatory response to wounding and seems to be involved in the resolution of wounding. We also have reported that the IL-24 protein is expressed in melanocytes and monocytes in the skin, and is upregulated by proinflammatory cytokines. Therefore, we propose that IL-24 functions to maintain homeostasis within the microenvironment of the skin during inflammation. We tested this hypothesis in two settings; wound repair and melanoma tumor cell migration. In vitro studies showed that cytokines involved in wound repair, most notably transforming growth factor (TGF)-α, upregulated IL-24 protein expression up to 5-fold in the immortalized keratinocyte cell line, HaCaT, and to a lesser extent in normal human epidermal keratinocytes (NHEKs). Examination of the function of IL-24 in both in vitro wounding and migration assays showed that IL-24 inhibits growth factor-induced proliferation and migration of NHEKs. Our earlier melanoma patient tumor data showing that loss of IL-24 expression is associated with melanoma tumor invasion, suggested that IL-24 inhibits migration and proliferation of melanocytes and developing melanoma tumors. To test this role, normal human epidermal melanocytes were treated with IL-24. Our results revealed that migration in response to TGF-α was decreased by 33%. In a similar manner when MeWo, a BRAF wild-type melanoma tumor cell line, was treated with IL-24 its migration in response to TGF-α was inhibited by 29%. The inhibition of melanoma migration appears to be, in part, through the downregulation of surface epidermal growth factor receptor expression as shown by the immunostaining of IL-24 – treated MeWo cells. We can conclude from these data that IL-24 is upregulated during inflammation and functions to inhibit growth factor-induced migration of keratinocytes, melanocytes, and some melanoma tumor cells.

Race Affects Clinical Responsiveness to Interferon-α in a Disease-dependent Fashion

*Zoltan Pos**, Silvia Selleri*, Tara L. Spivey*, Hui Liu*, Andrea Worschech*†, Marianna Sabatino*, Alessandro Monaco*, Andras Falus‡, Ena Wang*, Harvey J. Alter*, Francesco Marincola*. **Infectious Disease and Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD; †Genelux Corporation, San Diego Science Center, San Diego, CA; ‡Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary.*

Response to interferon alpha (IFNα) therapy in melanoma, CML, and HCV or HBV infections varies widely among individuals and is affected by many factors, including disease (sub) type and progression, overall disease burden, patient race, and the immune status of the host. Due to complex interactions among these factors, it is hard to identify independent genetic markers predicting responsiveness to IFNα in clinical cohorts. In this study, we sought to identify disease-independent genetic markers of IFNα responsiveness in healthy blood donors, with particular focus on race-associated response markers based on mounting evidence that chronically HCV-infected African Americans show sharply decreased responsiveness to IFNα compared with whites.

We analyzed T cells isolated from healthy whites and African Americans by comparing all STAT proteins affected by IFNα (STAT1, STAT2, STAT3, STAT4, and STAT5) in terms of protein expression, IFNα-induced phosphorylation, whole genome gene expression analysis of interferon-stimulated genes (ISGs), and comparison of about 1 million single nucleotide polymorphisms of the 2 ethnic groups. Surprisingly, we found that in contrast to HCV-infected individuals, healthy African Americans do not have impaired IFNα responsiveness either in terms of STAT activation, or IFNα-induced gene expression response. Although approximately 200 ISGs reacted to IFNα, we observed no significant differences in gene expression, activation, or in vitro response of ISGs to IFNα between healthy whites and African Americans. Only 1 gene was found to be affected by race (NUDT3, $P < 10^{-7}$), which, however, is not affected by IFNα, or known to be related to IFNα signal transduction, HCV infection, immunity against HCV, or disease progression. Although race-associated single nucleotide polymorphisms were found to be numerous, and many polymorphisms were associated with genes involved in IFNα signaling, these genetic differences did not affect responsiveness to IFNα in the absence of HCV infection.

We conclude that racial differences in IFNα responsiveness are related to polymorphisms affecting the interaction of the hosts with disease, rather than IFNα signaling per se. Hence, in chronic HCV infection, it seems that race affects an individual's ability to respond to the disease and its treatment by IFNα, independent of its effects on the therapy itself.

Modulation of Lymphocyte Function for Cancer Therapy: A Phase II Trial of Tremelimumab in Advanced Gastric and Esophageal Adenocarcinoma

*Christy Ralph**†, Eyad Elkord*, Fiona C. Thistlethwaite*, Debbie J. Burt*, Jackie O'Dwyer*, Peter L. Stern†, Robert E. Hawkins*. **Medical Oncology, Paterson Institute for Cancer Research, The Christie NHS Foundation Trust; †Immunology, Paterson Institute for Cancer Research, The University of Manchester, Manchester, United Kingdom.*

Purpose: Tremelimumab is a fully human monoclonal antibody, which blocks the cytotoxic T lymphocyte antigen 4 (CTLA4) and aims to inhibit T-cell regulation releasing useful antitumor immunity. Tremelimumab was investigated for efficacy, safety, and effect on T lymphocyte subsets in second-line treatment of advanced esophageal and gastric adenocarcinomas.

Design: Around 15 mg/kg tremelimumab was given intravenously every 3 months until symptomatic disease progression. Response was assessed clinically, radiologically, and by serial serum tumor-associated antigen (TAA) measurement. Lymphocyte phenotype, function, and cytokine production were characterized by flow cytometry, TAA lymphoproliferation to 5T4, and carcinoembryonic antigen and by cytokine enzyme-linked immunosorbent assay after polyclonal stimulation, pretreatment and at days 15, 30, 60, and 90 of each cycle.

Results: In all, 18 patients received a total of 32 cycles of treatment. Four had stable disease with clinical benefit. A single patient achieved a partial response after 8 cycles (25.4 mo) and continues to respond on study at 31.1 months. The most common drug-related toxicities were immune-mediated but mild: rash, diarrhea, and eosinophilia. There was a single toxic death due to bowel perforation complicating colitis; the other 2 toxicities of G3 or above were transient.

Pretreatment levels of natural T regulatory cells [$CD4^+CD25$high and either FoxP3 (forkhead box protein 3) or CTLA4] were 1.4% and showed an increase to 3% and 3.2%, respectively at day 15 ($P < 0.005$) before returning to baseline by day 60. In contrast, potential effector T cells ($CD4^+CD25$low/negative $CTLA4^+$) showed a sustained increase from 5% of lymphocytes at pretreatment to 8.9% to 10.4% from days 15 to 90 ($P < 0.005$). In all, 2 of 18 patients showed pretreatment lymphoproliferative responses to 5T4 pooled overlapping peptides, and 10 of 18 patients after

tremelimumab, at 16 of 94 time points (17%). In all, 9 of 10 patients showed a rise in proliferative response to carcinoembryonic antigen after tremelimumab ($P = 0.02$) and this change correlated with overall survival ($P = 0.002$). Pretreatment interleukin-2 release after polyclonal stimulation correlated with progression-free survival (Mantel Cox $P = 0.014$).
Conclusions: Although the objective response rate of tremelimumab was disappointing, 1 patient showed remarkably durable benefit. Interestingly, this patient developed posttreatment lymphoproliferative responses to both TAA after tremelimumab. Baseline immune responsiveness also seems necessary for treatment benefit suggesting potential for targeting of treatment.

Expression of the OX40 Costimulatory Receptor is Regulated Through a Dual TCR/Common Gamma Chain Cytokine-dependent Mechanism

William L. Redmond, Andrew D. Weinberg. *Cancer Research, Providence Portland Medical Center, Portland, OR.*
Ligation of several members of the tumor necrosis factor receptor superfamily, including OX40 (CD134) and 4-1BB (CD137), has been shown to provide critical signals that can augment T-cell differentiation and survival. Although OX40 engagement has been shown to greatly augment CD8 T-cell responses and antitumor immunity, the mechanisms regulating the expression of the OX40 receptor remain poorly understood. Earlier studies have suggested that T-cell receptor stimulation alone was sufficient to upregulate OX40 expression on T cells. However, in this study, we show that OX40 expression on CD8 T cells is regulated by a dual T-cell receptor and common γ chain cytokine-dependent pathway. Specifically, OX40 is expressed on CD8 T cells in an interleukin (IL)-2 and IL-4 – dependent manner that also requires signaling through the downstream kinase, JAK3. Importantly, combined therapy with an agonist, anti-OX40 mAb, and IL-2 cytokine-antibody complexes (IL-2c) restored the proliferative capacity of anergic tumor-reactive CD8 T cells in mice with long-term (> 40 d) well-established tumors, which was associated with the expansion of a granzyme B+/KLRG1low population of CD8 T cells. Additional studies are underway to determine the antitumor capacity of these cells and whether they represent effector and/or memory precursor cells. Together, these data show that dual anti-OX40/IL-2c therapy can restore the function of anergic tumor-reactive CD8 T cells and may represent a novel means of enhancing tumor-specific immunotherapy.

Cancer Immunotherapy Using Monocyte-derived DCs Stimulated With OK-432 and Prostaglandin E2 Pulsed With GP100 Epitope Peptide in Malignant Melanoma

Marimo Sato, Akira Kanamoto, Masahisa Jinushi, Hisako Katano, Kimiyasu Yoneyama, Akihiko Itoh, Hideaki Tahara. *Surgery and Bioengineering, Institute of Medical Science, The University of Tokyo, Minato-city, Japan.*
We have been involved in development of cancer immunotherapy using dendritic cells (DCs) to induce better immune responses. Our strategies include the usage of agents to induce desirable maturation of DCs in culture and better function in situ. To obtain DCs suitable for vaccination with class I-restricted melanoma-associated antigen gp100, we have been using monocyte-derived DCs stimulated with OK-432 and prostaglandin E_2 (OK-P-DCs). We have shown that OK-P-DCs have phenotypic characteristics of matured DCs, ability to successfully induce antigen-specific cytotoxic T lymphocytes in vitro, and capability to migrate (Sato M et al, *Cancer Sci*. 2003). On the basis of these preclinical results, we initiated the phase I clinical protocol to treat stage IV melanoma patients (n = 7) with OK-P-DCs pulsed with gp100 epitope peptide restricted to HLA-A*2402. In this study, we have evaluated peptide-specific immunologic responses in the enrolled patients using the methods established for the analysis of peripheral blood mononuclear cells. All the patients enrolled have well tolerated the treatment with no serious adverse events. The migration of the administered OK-P-DCs pulsed with gp100 was confirmed with the imaging for the radio-labeled DCs in the patients. Significant immune responses to gp100 were detected as early as 2 weeks after the first injection of OK-P-DCs pulsed with gp100 in all patients. These results warrant further development of our vaccination strategy using OK-P-DCs pulsed with gp100. The information related to these trials would be useful for developing effective immunotherapy against malignant melanoma.

Identification and Characterization of Novel HLA Class I Associated Peptides Presented Naturally on Lung Cancer Cells

Gomathinayagam Sinnathamby, Vivekananda Shetty, Punit Shah, Zacharie Nickens, Julie Hafner, Lisa Mariello, Ramila Philip. *Immunotope Inc, Doylestown, PA.*
Cancer of the lung is one of the most serious forms of cancer that kills over a million people worldwide every year. The National Cancer Institute estimates that there will be approximately 220,000 new lung cancer cases and 160,000 deaths in 2009 in the United States. Although significant advancements have been made in the understanding of the molecular carcinogenesis and treatment of lung cancer, most patients diagnosed with lung cancer eventually die owing to widespread metastases. Thus, there is a desperate need for the development of novel strategies to combat this form of cancer. Immunotherapeutic interventions to activate one's own immune system to fight against lung cancer are clearly very attractive. As cytotoxic T cells (CTL) play a critical role in antitumor immunity, identification, and characterization of peptide antigens displayed on cancer cells [human leukocyte antigen (HLA) class I epitopes] that activate cancer-specific CTLs are crucial to designing peptide-based T-cell vaccines. Toward this goal, we set to identify novel, naturally presented epitopes from 2 well-characterized lung cancer cell lines. Isolation of peptides presented by HLA class I molecules followed by mass spectrometry identified 230 HLA class I presented peptides. We selected 9 peptides based on the relevance of the source proteins in cancer progression and survival and confirmed their authenticity by using synthetic peptide analogs. These peptides also carry a strong consensus for HLA-A2 binding. CTL generated in vitro to these synthetic peptides not only recognized peptide-loaded targets but also lung cancer cells. Studies to characterize the source proteins and stability of the derived peptides when bound by HLA class I molecules are currently underway. Use of such naturally presented HLA class I epitopes as therapeutic vaccine is potentially a promising strategy to treat lung cancer.

The Effect of NK Cells on Lymphocyte Subpopulation in Cancer and Autoimmune Diseases

Ben-Ning Zhang*†, Masasi Irago*, Fei-Yan Lee*, Jian-Chuan Xia‡, Pei-Hong Wu‡, Yun-Kong Qi†, Huan Ren†, Naota Wantanabe§, Kazuteru Ukita*, Soukun Tsushimoto*, *Shimbu So**. **Kuramae Medical Clinic, Suiseikai Incorporated, Tokyo, Japan; †Immunology Department, Harbin Medical University, Harbin; ‡State Key Laboratory of Oncology in Southern China, Sun Yat-Sen University, Guangzhou, China; §Department of Pharmacology, Josai International University, Tokyo, Japan.*
There has been exact progression of cell immunotherapy in cancer treatment in these past 10 years. The Sun Yat-Sen University has done natural killer (NK) cell immunotherapy for a dozen years, finding that the combination of chemotherapy and immunotherapy achieves better survival rate compared with chemotherapy only (*J Immunother*. 31:63-71). To investigate the change of subpopulation of immune cells after NK cell infusion, we cultured autologous NK cells from healthy elderly people and patients with cancer or immune diseases and assayed the complete blood count, leukocyte demarcation, and lymphocyte subset. Regarding the healthy elderly people as standard, the normal ratio of granulocyte:monocyte:-lymphocyte is suggested to be 60%:5%:35% (SD = 0.007, 0.141, 0.007). Patients with cancer or immune diseases are biased to granulocytes ($P < 0.01$), suggesting the hyper-function of the sympathetic nervous system. After 1 round of NK cell infusion,

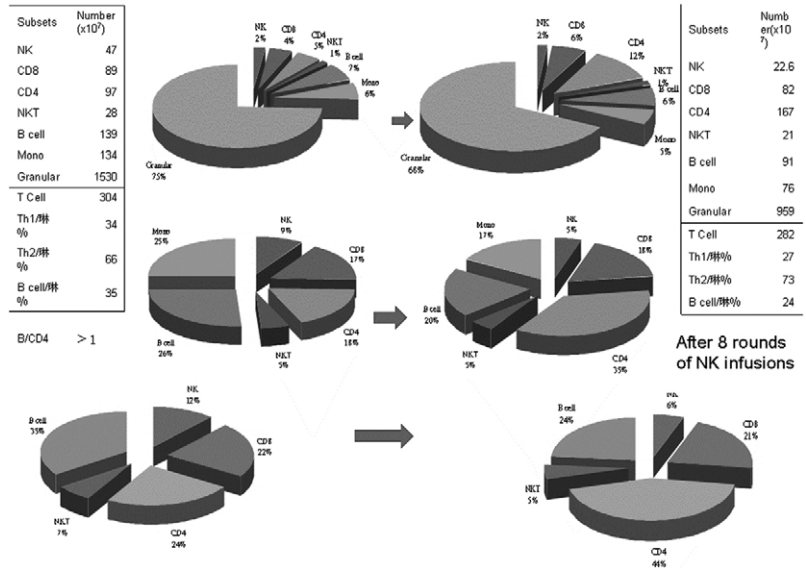

FIGURE 1 (So). The change of lymphocyte subsets after 8 rounds of NK cell infusion in a 48-year-old female with breast cancer.

increases of NK cells and enhancement of CD8 T cells in vivo were observed; after several rounds of NK cell infusions for a long time, the differentiation from CD4 T cells was presented, indicating the significance of CD4 T cells in maintaining the balance of the immune system. An improvement is seen on the abnormal B cell/CD4 T-cell ratio in autoimmune or allergic diseases with the NK cell infusions (Fig. 1), suggesting a new clinical reference system for diagnosis. It might be necessary to check the CD4 cell number for maintaining the healthy immune system and lightening the physical burden. Not only is it necessary to expect the effect of NK cell immunotherapy but also its reinforcement on the immune system. Immunotherapy is one more choice besides traditional cancer treatments: operation, chemotherapy, and radiotherapy, especially for patients who cannot be treated with or have no effect with them. We also recommend patients who just complete or are undergoing other treatments, to choose NK cell immunotherapy at the same time. Lastly, the observation that patients with chemicals may have irreversible damage on bone marrow function, because of low CD4 cell and low lymphocyte constitution, should not be neglected.

Update on the Predictive Nature of a Novel Melanocytic Gene Expression Signature for Outcome to High-dose Interleukin-2 Treatment in Patients With Advanced Melanoma

*Ryan J. Sullivan**, Yujin Hoshida†, Jean-Philippe Brunet†, Julie Aldridge‡, Steven Tahan§, Daniel Cho*, James W. Mier*, David F. McDermott*, Todd Golub†, Michael B. Atkins*. **Division of Hematology-Oncology; §Department of Pathology, Beth Israel Deaconess Medical Center; ‡Dana Farber Cancer Institute, Boston; †Broad Institute, Cambridge, MA.*

High-dose (HD) interleukin-2 (IL-2) remains 1 of 2 approved therapies by the Food and Drug Administration for the treatment of patients with metastatic melanoma (MM). We previously presented that overall and complete response (CR) rates in a contemporary series of patients with MM treated with HD IL-2, which are twice that reported in initial studies. In addition , we described that a novel melanocytic gene expression signature is associated with the treatment outcome. Clinical and radiologic data were collected and analyzed on 49 consecutive patients treated with HD IL-2 at BIDMC from October 2005 to October 2007. Response was evaluated via RECIST. Formalin-fixed paraffin-embedded (FFPE) tumor was obtained from consenting patients and classified as either class 1, defined by melanocyte-specific genes including MITF, or class 2, represented by immune genes, using the DNA-mediated annealing, selection, and ligation (DASL) technique. Two-sided Fisher exact test was used to compare the proportion of responses for patients in the 2 classes and Kaplan-Meier curves were generated to predict the overall and progression-free survival (PFS) in each of the 2 subclasses. In addition, reverse transcription-polymerase chain reaction will be performed on 35 patient samples using extracted RNA to determine B-raf mutational status. In all, 16 of 49 patients (32.6%) had a clinical response (partial response or complete response). FFPE tissue was obtained from 34 patients and 28 was subclassified into either class 1 (21) or class 2 (7). In all, 8 of 21 (38%) class 1 patients responded compared with 6 of 7 (86%) Class 2 ($P = 0.0768$) patients. In addition, the difference in 100-day, 1-year, and median PFS (the later 2.5 vs. 19.4 mo; $P = 0.03$) also favored those patients in the class 2 subgroup. The correlation of treatment outcome and classification with B-raf status is pending. Patients with tumors expressing an immune signature by DASL seemed more likely to respond to HD IL-2. In addition, patients in the class 2 subgroup had a superior PFS. Although these findings require prospective validation, they suggest that immune-related gene expression might contribute to IL-2 responsiveness.

Interferon-gamma Inhibits Renal Cell Carcinoma Growth by Limiting Polyamine Production and Increasing Nitric Oxide

*David J. Tate**, John R. Patterson*, Beatriz Finkel-Jimenez*, Arnold H. Zea*†‡. **Stanley S. Scott Cancer Center; †Microbiology, Immunology and Parasitology; ‡Section of Pulmonary and Critical Care Medicine, Louisiana State University, HSC, New Orleans, LA.*

Interferon-gamma (IFN-γ) has been referred to as a "master cytokine" due to its many effects, on cellular components during inflammation and immune responses. It plays a central role in intracellular homeostasis, as well as in host defense against a variety of microorganisms. In addition, it can have an active role in tumor regression. Patients with renal cell carcinoma (RCC) have few therapeutic options and their response to conventional therapy with interleukin-2 and IFN-α is only 10% to 20%. The treatment of advanced RCC with potent angiogenesis inhibitors and targeted agents has shown promising results. Our research approach is

directed to understanding the molecular and immunologic mechanisms by which IFN-γ alters the L-arginine metabolism through arginase and inducible nitric oxide synthase (iNOS) in RCC. The objective of this research is to determine the effect of IFN-γ in inducing iNOS/NO system as an advantage to promote tumor regression. Using murine and human cell lines of RCC with different arginase and iNOS expressions, we will determine how IFN-γ inhibits tumor growth by modulating the expression of arginase II, iNOS, nitric oxide, and polyamine production. Our results show that after the stimulation of the RCC cell lines with IFN-γ, significant increments of iNOS protein (Western blot) was observed after 24 hours in culture that decreased after 72 hours. However, as early as 6 hours the cell lines started to produce significant levels of nitrites (Greiss assay) compared with the untreated cells. After 24 hours, the level of arginase activity significantly decreased ($P = 0.03$) suggesting that such decreased activity may contribute to the antiproliferative effects triggered by IFN-γ. When we tested RCC proliferation (MTT assay) we observed a reduction ($P = 0.01$) on the RCC cell treated with IFN-γ compared with the untreated cells. The antiproliferative affect by IFN-γ is not due to apoptosis (flow cytometry) of the cells. We believe that the suppression of tumor growth is due to the low production of polyamines observed in the IFN-γ treated cells. The results indicate that IFN-γ is able to divert the L-arginine metabolic pathway toward the production of NO-inhibiting polyamineand stopping the growth of RCC cells. As immunotherapy may be one of the most promising approaches for treating RCC, understanding the mechanisms by which these tumors grow and the role of IFN-γ in antitumor responses may advance the development of combined therapies to improve the clinical outcome of patients with RCC.

In Vitro Immunization of Human Peripheral Blood Mononuclear Cells (PBMC) Using Targeted Activated T Cells to Induce Specific Primary Humoral Antibody Responses Against Breast Cancer

Archana Thakur, Oxana Norkina, Lawrence G. Lum. *BMT/Immunotherapy, Karmanos Cancer Institute, Detroit, MI.*

Purpose: The antitumor activity of the T cells is widely recognized but the role of humoral immune response in anticancer immunity has gained much less attention. Strategies to induce concerted cellular and humoral immune responses may provide a synergistic effect to augment antibreast cancer immunity. We developed an in vitro model to generate tumor-specific primary antibody response and approaches to enhance a protective antibody response that can be translated in clinical settings.

Methods: For the in vitro immunization model, we cocultured naive PBMCs, briefly exposed to SK-BR-3, with autologous irradiated Her2Bi-armed (*aATC) or unarmed activated T cells (*ATC) in the presence or absence of CpG-ODNs (5 mg/mL) for 14 to 21 days. Antibody synthesis was measured by whole-cell enzyme-linked immunosorbent assay, antibody-producing plasma cells by enzyme-linked immunosorbent spot or immunostaining. The cytotoxicity and cytokine profile was generated by the MTT assay and 25-plex human cytokine Luminex Array, respectively.

Results: A significantly increased ($P < 0.0001$) antibody was detected at day 14 in cocultures containing *ATC or *aATC and CpG (n = 9). Antibody levels were reduced by 20% to 50% after the serial absorption of culture supernatants to SK-BR-3 cells demonstrating specificity of the anti-SK-BR-3 antibody in cocultures with *ATC/aATC and CpG (n = 4). The anti-SK-BR-3 antibody showed increased CDC ($P < 0.02$) and ADCC ($P < 0.05$) in cocultures with *ATC/aATC and CpG. Cocultures containing *aATC and CpG showed significantly enhanced ($P < 0.001$) levels of IgG2 and significantly increased interleukin-13 ($P < 0.02$), interferon-γ ($P < 0.01$) and granulocyte macrophage-colony-stimulating factor ($P < 0.05$) cytokines. The immunization effect in this model was confirmed by coculturing SK-BR-3 lysate-loaded DCs with naive PBMC.

Conclusions: This is the first report showing in vitro antigen-specific primary antibody synthesis. It provides a platform for designing strategies to induce concerted cellular and humoral immune responses that may provide a synergistic effect to augment anticancer immunity.

Exploring the Immunogenicity of Dendritic Cells Pulsed With HLA-A2-Restricted Tumor Antigens in Colombian Individuals

Diana R. Tovar Murillo†, Rosalba Alfonso*, Carlos A. Parra-López†. *Group of Immunotherapy, Center for Immunological Studies on Clinical Onco-Immunology, PREVIMEDIC/Fundación Salud de los Andes; †Microbiología, Universidad Nacional de Colombia, Bogotá, Colombia.*

Despite vaccination with dendritic cells (DCs) pulsed with tumor-associated antigens, HLA-A2-restricted (TAAs-A2) has been thoroughly evaluated in white individuals as an alternative for cancer immunotherapy, the coverage and immunogenicity of this type of vaccine in Colombian population are yet to be established. In this study, the frequency of HLA-A2 alleles suitable for immunotherapy with TAAs-A2 and the inmunophenotyping of monocyte-derived DCs and TAAs-A2-specific CD8 T cells were examined in Colombian individuals.

Methods: A statistically significant sample of individuals representative of the Colombian population (180 individuals in the city of Bogotá) was selected to establish the frequency of the allele HLA-A* 02011 and HLA-A* 02013 using RPC-SSP . DCs obtained from peripheral blood monocytes of 24 individuals were purified using Rossette-Sep (StemCell Technologies) and the phenotype of DCs in response to the maturation cocktail [IL-6, IL-1b, TNF-a (CellGenix) and PGE-2 (Sigma)] was analyzed. To evaluate the immunogenicity of DC pulsed with TAA-A2 from HER-2/neu, NY-ESO1, Melan-A and Telomerase, CD8 T cells from normal individuals purified using magnetic beads (Miltenyi Biotech) were cocultured for 10 days in IL-2 and IL-7 and restimulated 2 days later with peptide-pulsed DCs. Production of interferon gamma (IFN-g), cytotoxicity and tetramer-positive CD8 T cells specific for TAA-A2 was assessed using flow cytometry.

Results: HLA typing evidenced an allelic distribution of HLA-A* 02011 and A*-02013 of 28%. Although upregulation of CD80, CD83, and CCR7 and down-modulation of CD209 were evidenced in response to maturation stimulus, no change in the expression of CD86 and HLA-DR in response to maturation was evidenced in DCs of the 24 individuals examined. Finally, the microculture system used allowed the detection and analysis of the response of CD8 T-cell precursors specific to TAA-A2 of HER-2, NY-ESO1, and Melan-A but not against telomerase.

Conclusion: The proved immunogenicity of DCs pulsed with TAA-A2 and the frequency of HLA-A2 alleles in the population led us to predict that DCs pulsed with TAA-A2 may be an important alternative for cancer immunotherapy in Colombia.

Evaluation of a Novel Human $CD4^+CD25DIMFOXP3^-$ T-Cell Population in Cancer Patients Undergoing Interleukin-2 Treatment

Todd A. Triplett†, Andrew Weinberg*,†. *MMI, Oregon Health and Science University; †Earle A. Chiles Research Institute, Portland, OR.*

Surface expression of the interleukin (IL)-2 receptor α-chain (CD25) has been used to identify $CD4^+CD25HiFOXP3^+$ regulatory T cells and activated T cells. However, new mouse antihuman CD25 monoclonal antibodies reveal a novel $CD4^+$ CD25DimFOXP3 − human T-cell population that expresses low levels of CD25, comprising mostly of $CD45RA^-CD95^+$ memory cells, and constitutes 15% to 80% of the human $CD4^+$ T-cell compartment. Specificity of the antibody was confirmed by $CD25^-$ Ig fusion protein. The function and role of this T-cell population in immune responses have currently not been determined. Therefore, we evaluated whether there was a change in this

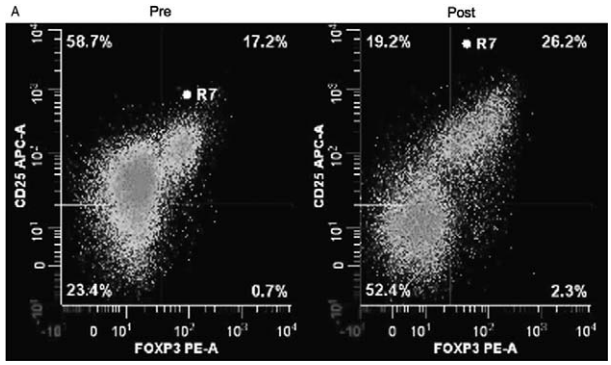

FIGURE 1 A (Triplett). CD3 $^{+}$CD4^{+} T cells from a patient before and 18 hours after initial infusion of IL-2.

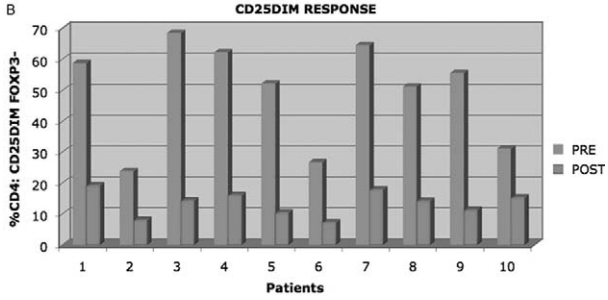

FIGURE 1 B (Triplett). Percentage of CD3^{+}CD4^{+} T cells that were CD25DIMFOXP3 − before and 18 hours after initial infusion of IL-2 in cancer patients.

population over the course of IL-2 treatment in cancer patients. Examination of peripheral blood mononuclear cells taken before and 18 hours after the initial infusions of IL-2 revealed that the percentage of CD4^{+} T cells that were CD25Dim was greatly diminished, whereas the percentage that was CD25^{-} increased and that was CD25HiFOXP3^{+} remained the same or increased. After 1 and 2 weeks of the last infusion, all 3 groups of the population returned to normal levels, although the CD25HiFOXP3^{+} population increased in some patients. Overall, our preliminary data suggest that the CD4^{+}CD25^{-}FOXP3 −, CD4^{+}CD25DimFOXP3 −, and the CD4^{+}CD25HiFOXP3 + T-cell populations respond differently from each other to IL-2 treatment in cancer patients and that the CD25Dim population may play a pivotal role in the immune responses of IL-2 patients against tumors (Fig. 1).

Immunization With Listeria Monocytogenes-based Vaccines Causes Pleotropic Effects on the Tumor Microenvironment

Anu Wallecha, Vafa Shahabi, John Rothman, Sandra Rivera, Paulo C. Maciag. *R and D, Advaxis Inc, North Brunswick, NJ.*

One of the major challenges in cancer immunotherapy is to overcome immunosuppression in the tumor microenvironment. We are developing the gram-positive bacteria *Listeria monocytogenes* (Lm) as a recombinant vector to induce T cell-mediated immunity to tumor-associated antigens (TAAs). By delivering the TAA as a fusion protein with a nonhemolytic fragment of the Lm virulence factor, listeriolysin-O (tLLO), the result is a more potent and broader T-cell response, which is able to eliminate the established tumors in mouse models. Although the expression of the TAA by the Lm vector and induction of specific T-cell responses are required for tumor regression, little is known about other properties of this strategy in modulating the tumor microenvironment. Using 2 different tumor models, TRAMPC-1 expressing human PSA (TPSA23) and NT-2 mammary carcinoma, we observed that immunization with an irrelevant Lm or a TAA-expressing Lm vaccine specific to the tumor type results in a considerable decrease in the frequency of such as CD4^{+}CD25^{+}FoxP3^{+} regulatory T suppressor cells (Tregs) and CD45^{+}CD11b^{+} cells in the tumor microenvironment when compared with untreated naive mice. A more significant decrease in Tregs and CD45^{+}CD11b^{+} cells was observed in regressing tumors from mice immunized with a TAA-specific vaccine compared with immunization with a control Lm vaccine that did not regress tumors. We also observed a significant increase in the frequency of $\gamma\Delta$ T cells infiltrating the tumors after vaccination with either control Lm or Lm-TAA vaccines. Further functional characterization of these cells is ongoing. These observations indicate that Lm expressing LLO fusion antigens may positively influence the tumor microenvironment by reducing the number of suppressor cells, in addition to generating potent specific T cell-mediate immune responses to TAAs and infiltration of other cytotoxic cells such as $\gamma\Delta$ T cells.

Immunization Against Weak Tumor Antigens: The Role of Peptide-Major Histocompatibility Complex Stability

*Alan M. Watson**, Megan Thompson†, Lawrence M. Mylin†, Todd D. Schell*. **Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey;* †*Department of Biological Sciences, Messiah College, Grantham, PA.*

CD8^{+} T-cell responses directed toward tumor self-antigens have been frequently investigated for use in tumor immunotherapy. However, CD8^{+} T cells targeting such epitopes are often limited by central and peripheral tolerance. In mice that develop tumors due to transgenic expression of Simian virus, 40 large tumor antigens (TAgs), CD8^{+} T cells targeting dominant TAg epitopes often exhibit tolerance while T cells targeting the subdominant site V epitope remain responsive. Site V was previously shown to form relatively unstable peptide-major histocompatibility complex (pMHC) complexes, suggesting that pMHC stability may contribute to the subdominant nature of site V and its decreased level of tolerance in tumor-bearing mice. In this study, we have identified mutations within site V that conserve epitope recognition and increase the stability of pMHC. These mutations also enhance site V immunogenicity. This shift in phenotype correlates with an increased window for T cells to encounter cross-presented antigen in vivo and results from increased T-cell accumulation compared with mice immunized with less stable site V variants. These results suggest that pMHC stability influences the T-cell immune response by altering the duration that naive T-cell responses can be initiated after cross-presentation. Novel immunization methods that extend the duration of cross-presentation for less stable T-cell epitopes may present an effective immunization strategy to elicit otherwise weak T-cell responses for tumor immunotherapy.

Successful Immunotherapy With IL-2/Anti-CD40 Directly Coincides With Specific Chemokine-mediated Mitigation of an Immunosuppressive Tumor Microenvironment

*Jonathan M. Weiss**, Timothy C. Back*, Anthony Scarzello*, Jeffrey J. Subleski*, Veronica L. Hall*, Jimmy K. Stauffer*, Dejan Micic*, Kory Alderson†, William J. Murphy†, Robert H. Wiltrout*. **Cancer and Inflammation Program, National Cancer Institute, Frederick, MD;* †*Dermatology, University of California, Davis, CA.*

Treatment of mice bearing established metastatic tumors with anti-CD40 antibodies resulted in only partial, transient antitumor effects, whereas combined treatment with interleukin (IL)-2 and anti-CD40 induced tumor regression. The antitumor effect of the treatment with anti-CD40 alone was qualitatively different from that mediated by the combined IL-2/anti-CD40 treatment, as it was dependent on CCR2-induced and monocyte chemoattractant

protein-1 – induced leukocyte recruitment into tumors. Unlike anti-CD40 alone, the effective antitumor therapy using IL-2/anti-CD40 was independent of CCR2 and was mediated by an augmentation of effector cell recruitment by interferonγ-induced chemokines concomitant with removal of immunosuppressive cells from the tumor microenvironment. Indeed, the combined IL-2/anti-CD40 treatment caused an interferonγ-dependent reduction in $CD4^+$/FoxP3+ Tregs, myeloid-derived suppressor cells and T_H2 chemokine expression in the tumor microenvironment that was not observed after treatment with anti-CD40 alone. In addition, IL-2/anti-CD40 combined treatment induced significant infiltration of established tumors by $CD8^+$ T cells in association with an increase in T_H1 chemokine expression. Interestingly, IL-2/anti-CD40 induced an increase in peripheral Tregs that did not prevent the antitumor response, suggesting that only the redistribution of effector and regulatory leukocytes within the tumor microenvironment was predictive of therapeutic success. The therapeutic efficacy of anti-CD40 may be limited by its inability to overcome immunoregulatory effects within tumors, and its synergistic combination with IL-2 not only resulted in potentiation of the antitumor effect of both treatments, but elicited novel antitumor mechanisms that were not induced by either treatment alone. Thus, the combined IL-2/anti-CD40 treatment holds substantially more promise for clinical cancer treatment.

Activation of Antitumor Functions of Natural Killer Cells And Suppression of Pro-tumor Functions of Monocytes by Lenalidomide

*Yibing Xu**, Hong-wei Wu*, Jianping Sun*, Robert C. Seeger*,†. **Center for Cancer and Blood Diseases and Saban Research Institute, Childrens Hospital Los Angeles; †Keck School of Medicine, University of Southern California, Los Angeles, CA.*

Background: Over the past 20 years, long-term survival for patients with high-risk neuroblastoma has improved to 40% with intensive nonspecific cytotoxic induction and consolidation therapy, and with 13-cis retinoic acid$^+$antitumor cell monoclonal antibody (mAb) (anti-GD2 ch14.18$^+$IL-2$^+$GM-CSF) therapy for minimal disease after consolidation. We hypothesize that survival can be further improved with immunotherapy that maximizes natural killer (NK)$^+$antitumor mAb cytotoxicity and cytokine/chemokine secretion while suppressing monocyte/macrophage pro-tumor effects. The aims of this research are to determine the ability of lenalidomide (Revlimid®) (1) to activate NK antitumor functions (direct cytotoxicity, ADCC , and secretion of cytokines); (2) to overcome the suppression of NK activation by interleukin (IL)-6 and transforming growth factor-β1; (3) to decrease monocyte secretion of IL-6 in response to neuroblastoma cells; and (4) to inhibit monocyte stimulation of neuroblastoma cell proliferation.
Methods: Purified NK cells from normal adults were activated with IL-2$^+$lenalidomide$^+$CD16/FcγRIII stimulation for 24 to 72 hours. Direct cytotoxicity and ADCC with mAb ch14.18 were quantified after 72 hours of activation by coculturing NK cells with calcein-AM labeled neuroblastoma cells for 6 hours and then quantifying loss of calcein from target cells. Cytokine release was quantified after 24 to 72 hours with the BD cytometric bead array assay or with enzyme-linked immunosorbent assay assays for individual cytokines.
Results: Activation of purified NK cells for both direct cytotoxicity and ADCC (with mAb ch14.18) against drug-sensitive and multidrug-resistant neuroblastoma cell lines was significantly increased by adding lenalidomide to IL-2. Addition of lenalidomide also increased NK secretion of IL-2, granulocyte macrophage-colony stimulating factor, interferon-γ, tumor necrosis factor-α, MIP1α, MIG, release of granzyme A and B, and synthesis of perforin, but decreased secretion of IL-6 and IL-10. IL-6, IL-6+sIL-6R, and transforming growth factor-β1 suppressed IL-2+anti-CD16 activation of NK secretion of interferon-γ, but lenalidomide reversed this immunosuppression. Conditioned medium of neuroblastoma cell lines stimulated purified blood monocytes to secrete IL-6, and lenalidomide significantly inhibited secretion of IL-6. Finally, monocytes stimulated proliferation of neuroblastoma cell lines, and this was inhibited by lenalidomide.
Conclusions: Lenalidomide significantly increases antitumor cytotoxicity and cytokine/chemokine secretion by NK cells and inhibits immunosuppressive and tumor cell proliferative effects of monocytes. These data support clinical testing of lenalidomide with antitumor cell mAbs in patients with recurrent, high-risk neuroblastoma.

An Effective Interleukin-2 Cancer Immunotherapy Delivered by a Novel Polymeric Nanoparticle in Melanoma

*Hong Yao**†, Ming Li*, Samuel S. Ng*, Marie C. Lin*†. **Chemistry, The University of Hong Kong, Hong Kong, China; †Biomedical Engineering Research Centre, Kunming Medical University, Kun Ming, China.*

Interleukin-2 (IL-2) has been shown to possess antitumor activity in numerous preclinical and clinical studies. However, the short half-life of recombinant IL-2 protein in serum requires repeated high doses administration, resulting in severe side effects. Although adenovirus-mediated hIL-2 gene therapy has shown antitumor efficacy, the host antibody response to adenoviral particles and potential biosafety misgivings prevented its clinical application. Here we report a novel polymer, consisting of low-molecular weight Polyethylenimine (PEI 600 Da) linked by Cyclodextrin and conjugated with Folate (referred to as H1). The H1 assembled hIL-2 plasmid to form H1/phIL-2 polyplexes with size around 100 nm. Peritumoral injection of these polyplexes suppressed the tumor growth and prolonged the survival in C57/BL 6 mice bearing B16-F1 cells. Importantly, the antitumor effects of H1/hIL-2 plasmid (DNA 50 μg) were similar to those of Adv-hIL-2 (2×108 pfu). Furthermore, we showed that this treatment activated immune cells such as cytotoxic T lymphocyte and natural killer cells. Interestingly, it has no effect on the number of $CD4^+CD25^+$ Treg cells of peripheral blood, which was reported to suppress antitumor immune responses. In conclusion, these results showed that H1/phIL-2 polyplexes are an effective and safe therapy with an efficacy comparable to that of Adv-hIL-2. This treatment represents an alternative gene therapy strategy for melanoma.

Intratumoral Immunotherapy With Chitosan/IL-12 Eradicates Established Tumors and Elicits Durable, Tumor-specific Immune Responses

*David A. Zaharoff**, Kenneth W. Hance†, Connie J. Rogers†, Jeffrey Schlom†, John W. Greiner†. **Biomedical Engineering, University of Arkansas, Fayetteville, AR; †Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, MD.*

Interleukin (IL)-12 is a potent antitumor cytokine with significant clinical toxicities during systemic immunotherapy. In an effort to reduce clinical toxicities while improving the antitumor efficacy of IL-12, we have developed a novel biomaterials-based delivery system for local administration. Our data published earlier were the first to show that we can enhance the immunomodulatory activity of a recombinant cytokine (GM-CSF) through coformulation with chitosan—a natural, biodegradable polysaccharide derived from the exoskeletons of crustaceans. Here, we hypothesized that intratumorally administered coformulations of chitosan and IL-12 (chitosan/IL-12) could similarly enhance the immunomodulatory and antitumor activity of IL-12 while limiting its systemic exposure. Noninvasive in vivo imaging revealed that IL-12 in saline disseminated quickly and became undetectable between 24 to 48 hours after intratumoral injection. In contrast, IL-12 could be measured for up to 6 days when coformulated with chitosan. In antitumor studies, 3 weekly intratumoral injections of IL-12 (1 μg) in saline modestly delayed the growth of established subcutaneous

MC32a tumors in carcinoembryonic antigen-transgenic mice, with only 10% of treated tumors completely regressing. In contrast, intratumoral immunotherapy with chitosan/IL-12(1 μg) induced durable, complete tumor regressions in 80% to 100% of mice. In a second tumor model, 100% of subcutaneously implanted pancreatic tumors (Panc02) were eradicated after 2 to 3 intratumoral administrations of chitosan/IL-12(1 μg). Depletion of $CD4^+$ or Gr-1+ cells had no impact on chitosan/IL-12–mediated tumor regression. However, $CD8^+$ cell or natural killer cell depletion completely abrogated antitumor activity, thus implying essential roles for these immune subsets. Intratumoral chitosan/IL-12 immunotherapy was found to generate systemic tumor-specific immunity, as more than 80% of mice cured with intratumoral chitosan/IL-12 immunotherapy were at least partially protected from tumor rechallenge at a distal site. Furthermore, cytotoxic T lymphocytes isolated from spleens of cured mice were found to lyse MC32a targets and gp70 peptide (p15E604-611)-loaded targets at levels of up to 50%. Gp70 is an endogenous retroviral glycoprotein commonly overexpressed in murine tumors, including MC32a. In summary, chitosan/IL-12 immunotherapy can (i) retain IL-12 in the tumor microenvironment, (ii) eradicate established colorectal and pancreatic tumors, and (iii) generate systemic tumor-specific immunity capable of inhibiting tumor recurrence. At this dose/schedule, we found chitosan/IL-12 to be a well-tolerated, effective immunotherapy that deserves further consideration for testing in humans.

ENHANCING CANCER VACCINES/ COMBINATIONS

Androgen Ablation Augments Cellular Immune Responses to PSA in a Humanized PSA/HLA-A2.1 Transgenic Mouse Model

*M. Simo Arredouani**, Stephanie Tseng-Rogenski†, Brent Hollenbeck†, Deborah Defeo-Jones‡, Clara Hwang†, Martin G. Sanda*. **Surgery, BIDMC/Harvard Medical School, Boston, MA; †Urology, University of Michigan School of Medicine, Ann Arbor, MI; ‡Merck Laboratories, Whitehouse Station, NJ.*

Background: Immunologic tolerance to tumor-associated antigens (TAA) poses a significant obstacle to effective, TAA-targeted immunotherapy, imposing the need for a combination of cancer vaccines with other treatments that would circumvent immune tolerance. Among these treatments, androgen deprivation is gaining momentum.

Hypothesis: We sought to investigate whether androgen deprivation in mice would result in circumventing immune tolerance to prostate TAA by impacting CD8 cell responses.

Methods: To this end, we generated a transgenic mouse that expresses human prostate-specific antigen (PSA) specifically in the prostate, and crossed it with the HLA-A2.1 transgenic mouse. The hybrid mouse was used in immunization experiments.

Results: The PSA transgenic mouse showed restricted expression of PSA in the prostate and detectable circulating PSA levels. In addition, PSA expression was androgen-dependent with reduced PSA expression in the prostate within 1 week of castration, and undetectable PSA by day 42 after castration as evaluated by enzyme-linked immunosorbent assay. Castration of male PSA/HLA-A2.1 hybrid mice before immunization with a PSA-expressing recombinant vaccinia virus resulted in a significant augmentation of PSA-specific cytotoxic lymphocytes. This increase was observed for both interferon-γ release and tetramer staining levels after restimulation of splenocytes with PSA-derived HLA-A2.1–restricted peptides or full recombinant PSA.

Conclusions: This humanized hybrid mouse model provides an adequate system to gain additional insight into the mechanisms of immune tolerance to PSA, and to test novel strategies aiming at circumventing immune tolerance for the benefit of PSA and other TAA-targeted prostate cancer immunotherapies in prostate cancer patients.

Safety Data of Montanide ISA 51VG and Montanide ISA 720VG in Human Therapeutic Vaccines

Stephane Ascarateil, Héloïse Imbault. *AVI, SEPPIC, Paris, France.*

Montanide ISA 51VG and Montanide ISA 720VG are adjuvants rendering stable water in oil emulsions when mixed with antigenic media. Montanide ISA 51VG is based on a blend of mannide monooleate derivative surfactant and mineral oil, whereas Montanide ISA 720VG uses nonmineral oil. A review of safety data obtained for both adjuvants during their development is conducted. Preclinical safety information includes a histologic injection site study after injection of placebo emulsion into guinea pig muscles. Observation of inflammation, granulomas and necrosis and presence of exogenous material are reported and commented in vaccinated muscle compared with controls. Safety data reported in published clinical trials in human up to 2008 are also commented. Local and general adverse events observed with Montanide ISA 51VG in cancer but also therapeutic AIDS vaccines are listed for more than 5000 patients. Mainly mild or moderate reactions were recorded and described as flu-like symptoms and erythemas. Equivalent scores were reported with Montanide ISA 720VG in malaria and cancer mainly, although in fewer trials and patients.

How to Exploit the Immunogenicity of Chemotherapy or Radiotherapy to Achieve a Personalized Therapy of Cancer

Laurence Zitvogel*, *Laetitia Aymeric**, *Lionel Apetoh†, Yuting Ma**, *François Ghiringhelli†, Antoine Tesniere‡, Guido Kroemer‡. *Department De Biologie Clinique; †Gustave Roussy Institute; ‡INSERM, Villejuif, France.*

Conventional cancer therapies rely on radiotherapy and chemotherapy. Such treatments supposedly mediate cancer effects through the direct elimination of tumor cells. However, such anticancer therapies can also modulate the host immune system in several ways. Drugs can inhibit immunosuppressive pathways, activate distinct immune effectors, sensitize tumor target cells to cytotoxic T lymphocyte attack, or generate an immunogenic cell death modality, all culminating in eliciting or enhancing anticancer immune responses contributing to the tumoricidal activity of the drug. Indeed, we reported that anthracycline-mediated cell death is immunogenic in tumor-bearing hosts through a molecular pathway involving membrane exposure of calreticuline by tumor cells.[1–3] Calreticuline is mandatory for the uptake by dendritic cells of dying tumor cells. More generally, anthracyclines, x-rays, and platinum based-therapies mediate a tumoricidal activity relying on $CD8^+$ T cells, $CD11c^+$ dendritic cells, and the IFNg/IFNgR signaling pathway, but not interleukin-12. We determined which biochemical or metabolic components expressed or released by dying tumor cells could trigger the immune system and participate in the immunogenicity of cell death. While HMGB1/TLR4 are mandatory for the processing of dying bodies by dendritic cell and the activity of chemotherapy, other components such as the inflammasome complex NLRP3 recently unraveled will be presented at the meeting. Moreover, other innate components seem to be involved in the immunogenicity of chemotherapy, such as NKT and gamma delta T cells. These results delineate a clinically relevant immuno-adjuvant pathway triggered by tumor cells. Designing an algorithm of immune parameters dictating the success of chemotherapy becomes possible. The clinical implementations of this work in the management of breast cancer will be detailed.

References:

1. Obeid M, et al. Calreticuline exposure dictates the immunogenicity of anthracyclines. *Nat Med.* 2007;1:54–61 [Epub ahead offprint Dec 24, 2006].
2. Zitvogel L, et al. Cancer in spite of immunosurveillance. Immunosubversion and immunosuppression. *Nat Rev Immunol.* 2006:715–727.
3. Casares N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med.* 2005;202:1691–1701.

4. Apetoh L, et al. TLR4 -dependent contribution of the immune system to the antitumor effects of chemotherapy and radiotherapy. *Nat Med Aug*. 2007.

Eradication of Established Tumors by Administration of a Cellular Vaccine Secreting an APC-specific CTLA-4-ERBB2/HER2 Fusion Protein

Ute Burkhardt, Arjen Sloots, Annemarie Schimpf, Thorsten Geyer, Winfried S. Wels. *Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt, Germany*.

Presentation of tumor-associated antigens by professional antigen-presenting cells (APC), in particular by dendritic cells, is critical for the induction of tumor-specific T-cell responses. To facilitate targeted delivery of a tumor antigen to APCs in vivo, we generated a plasmid DNA vaccine that encodes a secreted fusion protein consisting of the extracellular domain of cytotoxic T lymphocyte antigen (CTLA)-4 for binding to B7 molecules on APCs, fused to residues 1 to 222 of ErbB2/HER2 as an antigenic determinant. The ErbB2 receptor tyrosine kinase transmits important growth and survival signals, and is overexpressed by many human tumors of epithelial origin. Chimeric CTLA-4-ErbB2 protein expressed from the DNA construct displayed specific binding to B7-expressing cells and subsequent cellular uptake in vitro. Induction of humoral and cellular immune responses and antitumoral activity of the DNA vaccine were tested in murine tumor models with transfected renal carcinoma (Renca) cells expressing the respective antigen. Vaccination of BALB/c mice with CTLA-4-ErbB2 plasmid DNA markedly improved tumor-free survival upon challenge with ErbB2-expressing Renca cells in comparison with untargeted ErbB2 or a control CTLA-4-NY-ESO1 DNA vaccine, accompanied by induction of stronger ErbB2-specific antibody and CTL responses. Furthermore, antitumoral activity of such a CTLA-4 fusion vaccine could be reproduced in immunotolerant BALB-neuT mice, where a corresponding CTLA-4-Neu (residues 1 to 224) markedly delayed the onset of spontaneous Neu-driven mammary carcinomas (Sloots, et al. *Clin Cancer Res*. 14:6933-6943, 2008). For immunotherapy of established tumors, we generated a syngeneic cell line continuously secreting the CTLA-4-ErbB2 vaccine to achieve high local concentrations upon implantation of these nontumorigenic bystander cells in the tumor vicinity. Therapeutic vaccination of tumor-bearing mice by inoculation of such cells close to subcutaneously growing Renca-lacZ/ErbB2 tumors resulted in complete tumor rejection in the majority of animals. accompanied by induction of ErbB2-specific antibody and T-cell responses. Furthermore, long-term protection was induced, indicated by the rejection of a lethal dose of systemically applied Renca-lacZ/ErbB2 tumor cells by vaccinated animals 2 months later. Our results show that potent immune responses and antitumoral activities can be induced by in vivo expression of tumor-associated antigens targeted to APCs, thus providing a rationale for further development of this approach for specific cancer immunotherapy.

Dendritic Cell Redirection of Regulatory T-cell Responses to Ovarian Tumor Antigens

*Martin J. Cannon**, Timothy J. O'Brien*, Maurizio Chiriva-Internati†. **University of Arkansas for Medical Sciences, Little Rock, AR; †Texas Tech University Health Sciences Center, Lubbock, TX*.

Accumulating evidence has clearly shown that the distribution of self-reactive high-affinity T-cells is heavily skewed in favor of the $CD4^+$ T regulatory (Treg) compartment, as a result of thymic selection that favors differentiation of autoreactive T-cells to become Treg rather than autoimmune effector T-cells. In addition, self-antigens drive peripheral expansion of Treg, and direct their accumulation in sites where the antigen is processed and presented. In the face of this reality, it is unsurprising that tumor antigen-loaded mature dendritic cells (DC) activate and expand $CD4^+$foxp3+ Treg in vitro, supporting the clinical observation that vaccination with cytokine-matured DC expands $CD4^+$foxp3+ Treg in cancer patients. It is thus probable that DC activation of antitumor effector T-cell responses would be seriously compromised by concomitant activation of Treg. For DC vaccination to be clinically effective, DC need to access the Treg repertoire of self-reactive T-cells and redirect differentiation toward effector T-cell responses. We present innovative results showing that treatment of DC with interleukin (IL)-15 and a p38 MAPK inhibitor offers potent synergy in antagonism of $CD4^+$ Treg differentiation and redirection toward ovarian tumor antigen-specific Th17 responses. Although treatment of DC with p38 MAPK inhibitor alone reduced the $CD4^+$ foxp3+ T-cell response, potentiation of effector Th17 $CD4^+$ T-cell responses and activation of tumor antigen-specific $CD8^+$ cytotoxic T lymphocyte responses was not observed unless DC were additionally treated with IL-15. Functional analysis revealed further benefits to IL-15/p38 MAPK inhibitor treatment of DC, including (1) reduced expression of B7-H1, and of indoleamine 2,3 dioxygenase, both of which offer possible mechanisms for reduced recruitment and activation of $CD4^+$ foxp3+ Treg by mature DC; (2) elevated CCR7 expression by DC, which may enable homing to draining lymph nodes; (3) diminished $CD4^+$ T-cell expression of CTLA-4 and PD-1, both of which may reduce susceptibility to apoptosis or anergy in the tumor microenvironment; and (4) $CD4^+$ T-cell expression of CCR4 and CXCR4, and strong chemotactic responses across vascular endothelium in response to CCL22 and CXCL12, suggesting the potential for ovarian tumor infiltration. Recent experimental and clinical evidence that Th17 responses are associated with strong antitumor immunity stands in sharp contrast to the known association of Treg infiltration with increased morbidity and mortality in ovarian cancer, and clearly points to the potential clinical benefit to be gained from therapeutic strategies that redirect Treg responses to Th17 responses.

Sperm Protein 17 Vaccination Induces a Long-term Protective Immune Response Against Ovarian Cancer

*Maurizio Chiriva-Internati**, Yu Yuefei*, Marjorie Jenkins†, Martin Cannon‡, Caroline Chapman§, Everardo Cobos*, W. Martin Kast‖. **Division Hematology/Oncology TTUHSC, Lubbock; †Departments of Internal Medicine and Obstetrics & Gynecology, and the Laura W. Bush Institute for Women's Health and Center for Women's Health and Gender-Based Medicine, TTUHSC, Amarillo, TX; ‡Microbiology and Immunology, UAMS, Little Rock, AR; ‖Molecular Microbiology & Immunology and Obstetrics & Gynecology, Norris Comprehensive Cancer Center, University of Southern California, USC, Los Angeles, CA; §Division of Breast Surgery, The University of Nottingham, Nottingham, Nottingham, United Kingdom*.

Sperm protein (Sp17) is an attractive antigen to target using therapeutic vaccines because of its overexpression in ovarian cancer, especially in metastatic tissues, and its limited expression in other organs. Our studies offer the first evidence that an Sp17-based vaccine can induce long-term protection against ovarian cancer development in C57BL/6 mice with ID8 murine ovarian cancer cells. Six-week-old female C57BL/6 mice were vaccinated with Sp17 protein and CpG strategy. Our results show the induction of an immune response against Sp17 protein that is mediated primarily by CD8 T cells. Vaccination induced major histocompatibility complex class I expression and cytokine production [interferon-γ, tumor necrosis factor-A, interleukin 2 (IL-2), IL-4, and IL-5] within ovarian cancer (OC). Sp17-vaccinated C57BL/6 mice had an 85% survival rate at 10 months of age. In contrast, all control mice succumbed to OC or had heavy tumor loads. Crucially, this long-term protective immune response was not associated with any measurable induction of autoimmunity. Inducing long-term protection against OC by vaccination at the earliest signs of its development has the potential to cause a dramatic paradigm shift in the treatment of this disease.

E6020, a Novel Immunoadjuvant, is Effective at Stimulating HER2/NEU Cancer Immunity in Healthy Donors and Breast Cancer Patients

*Guy T. Clifton**, Kathy Ciano†, Athina Zacharia†, Mohmed Mursal†, Yusuf Jama†, Sally Ishizaka‡, Lynn Hawkins‡, George E. Peoples*, Sathibalan Ponniah†. **General Surgery, Brooke Army Medical Center, Fort Sam Houston, TX; †Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD; ‡Eisai Research Institute, Andover, MA.*

Background: Cancer vaccine formulations need effective immunoadjuvant/s to overcome immune tolerance and generate robust antitumor immune responses. E6020 is a synthetic, attenuated toll-like receptor-4 agonist that exhibits immunostimulatory properties in mice. E6020 stimulates secretion of cytokines (tumor necrosis factor-α, interleukin-6, and interleukin-1β) and stimulates dendritic cells to enhance immune response. The objective of this study was to evaluate E6020 as a potential immunoadjuvant for cancer vaccines in human peripheral blood mononuclear cells (PBMCs).

Methods: PBMC samples from breast cancer patients (BCPs) enrolled in peptide-based cancer vaccine trials or healthy donors (HDs) were stimulated in the presence and absence of E6020 (provided by Eisai Research Institute, MA) or a control, molecule ER-804053 (a diastereomer of E6020 showing reduced in vitro potency). In addition, the PBMCs from the same patients were also stimulated with the Flu-M peptide (viral antigen) or cancer vaccine peptides, E75 (HER2/neu 369-377) and GP2 (HER2/neu 654-662) without adjuvant (no adjuvant, NA) and with E6020 (experimental adjuvant, EA) or ER4053 (control adjuvant, CA) at various concentrations. Functional immune responses were measured using an ex vivo interferon-γ enzyme-linked immunosorbent spot assay.

Results: PBMC samples from 4 HD and 17 BCP were evaluated. In the absence of any added peptide the HD responses were 1 ± 0.4 spots (NA), 26 ± 3.7 (EA), and 0.3 ± 0.3 spots/106 cells (CA) ($P = 0.03$). BCP responses were 0.2 ± 0.1 spots (NA), 5.5 ± 2.5 spots (EA), and 0.2 ± 0.13 spots/106 cells (CA) ($P = 0.04$). With Flu-M peptide, the enzyme-linked immunosorbent spot responses increased from 1 ± 0.6 spots (NA) to 29.3 ± 5 spots (EA) compared with 2.5 ± 1 spots/106 cells (CA) in HD ($P = 0.02$) and increased from 2.3 ± 0.8 spots (NA) compared with 5 ± 2 spots (EA) and 1 ± 0.3 spots/106 cells (CA) in BCP ($P = 0.05$). HD PBMCs incubated with E75 had 1 ± 0.7 spots (NA), 21 ± 5.5 spots (EA), and 1.5 ± 0.9 spots (CA) ($P = 0.05$), whereas PBMCs incubated with GP2 produced 1 ± 0.6 spots (NA), 17.8 ± 4.6 spots (EA), and 1.5 ± 0.3 spots/106 cells (CA) ($P = 0.04$). In BCP, PBMCs incubated with E75 produced 1.0 ± 0.8 spots (NA), 5.3 ± 1.8 (EA), and 0.5 ± 0.2 spots/106 cells (CA) ($P < 0.01$). BCP PBMCs incubated with GP2 produced 1.8 ± 1 spots (NA), 5.6 ± 1.8 spots (EA), and 0.9 ± 0.3 spots/106 cells (CA) ($P = 0.02$).

Summary: E6020 is an effective nonspecific, viral peptide, and cancer peptide-specific immunostimulant in HDs and cancer patient PBMCs. These findings suggest the potential use of E6020 as an immunoadjuvant in cancer vaccines.

Self-renewing, Proliferating Autologous Melanoma Cells, Alone or Combined With Dendritic Cells, as Vaccine Immunotherapy for Patients With Metastatic Melanoma: Results of Consecutive Phase II Trials

Robert O. Dillman, Senthamil R. Selvan, Andrew N. Cornforth, Carol DePriest, Cristina DeLeon, Cheryl Mayorga, Patric M. Schiltz, Linda D. Beutel. *Hoag Cancer Center, Newport Beach, CA.*

Background: Patient-specific vaccines using proliferating tumor cells, which may represent tumor stem cells, may be ideal for inducing an immune response to the cells responsible for the generation of new metastatic sites of melanoma. Such a response could enhance survival.

Methods: In sequential phase II trials, patients were treated with vaccines derived from autologous continuously proliferating tumor cells (TCs) or dendritic cells (DCs) loaded with TCs. Short-term continuous cell lines were established from surgically excised depots of metastatic melanoma, expanded to 200 million cells, irradiated, and cryopreserved. In the second trial, autologous DCs were derived from peripheral blood mononuclear cells by culturing in interleukin-4 and granulocyte macrophage-colony stimulating factor (GM-CSF). DCs were incubated with the irradiated TCs to create the vaccine. In the TC vaccine trial patients, most patients received injections of interferon-γ and/or GM-CSF as an adjuvant. In the DC vaccine trial, each vaccine dose was suspended in 500-μg GM-CSF for injection. In both the trials, the treatment plan consisted of subcutaneous injections weekly $\times 3$, then monthly $\times 5$.

Results: During 1990 to 2000, 78 metastatic melanoma patients received the TC vaccine; during 2001 to 2006, 56 patients were enrolled to receive the DC vaccine. There were 74 eligible and evaluable patients in the TC trial, and 54 in the DC trial. Median age was 50 years and a mean of 6.7 vaccinations were administered in both the trials. Treatment was administered in the Hoag Cancer Center for only 47% of TC patients, but for 100% of DC patients. Both vaccines were well tolerated. At the time when vaccine therapy was initiated, 53% and 28% of patients had measurable disease, with objective response rates of 7.7% and 0% for the TC and DC trials, respectively. In the TC trial, 18% of 39 patients with measurable disease had either an objective response or survived at least 3 years from the start of vaccine treatment, whereas in the DC trial 40% of 15 patients survived at least 3 years from the start of treatment. Five-year survival rates were 29% and 54% in the TC and DC trials, respectively. In a comparison of 48 TC patients who received at least 3 vaccinations to the 54 DC patients, the latter group still had superior survival ($P = 0.016$ log rank).

Conclusions: These patient-specific vaccine approaches are feasible, safe, associated with encouraging survival, and warrant further investigation. Long-term survivors were documented in both the trials. On account of differences in patient characteristics, it is unclear whether the DC vaccine is truly associated with superior outcomes.

Sustained Activation of INKT Cells Associated With Low-Dose Cyclophosphamide Treatment Leads to a Synergistic Inhibition of Established Tumors

Stéphanie Corgnac*, Sophie Sierro†, Kathrin Stirnemann*, Jean-Pierre Mach*, Pedro Romero†, *Alena Donda**. **Department of Biochemistry, University of Lausanne; †Lausanne Branch, Ludwig Institute for Cancer Research, Lausanne, Switzerland.*

We have previously shown that the administration of recombinant αGalCer/CD1d complexes leads to sustained activation of invNKT (iNKT) and natural killer (NK) cells, resulting in a potent antitumor effect, when CD1d was targeted at the tumor site by its genetic fusion to an anti-HER2 scFv fragment. Here, we show that the efficacy of this immunotherapy strategy can be further enhanced by its combination with a low dose of cyclophosphamide (CY). In this context, untargeted soluble αGalCer/CD1d combined with CY also leads to potent antitumor effect, whereas each regimen alone had no effect. These new data may allow extending iNKT-mediated antitumor therapy to most cancers for which no surface antigen and/or tumor-specific scFv are available for CD1d targeting. Alternating treatment of CY (0.5 mg intraperitoneal) and αGalCer/CD1d (25 μg intravenous), was started on palpable tumors and repeated at 3 to 4 days interval. Potent tumor inhibition was obtained in various models of grafted tumors including B16 melanoma, MC38 colon carcinoma, or EL4 lymphoma cell lines, as well as in a spontaneous melanoma model of Tyr:Nras transgenic mice. In these mice, in addition to growth inhibition of the existing tumors, the appearance of new nodules was blocked by the combined treatment. The mechanisms of the sustained activation of iNKT and NK cells by recombinant αGalCer/CD1d proteins, compared with their well-reported unresponsiveness induced by αGalCer alone, are investigated. In addition, current analyses of lymphoid organs and tumor tissue may shed light on the CY-mediated effects, in particular on the tumor immunosuppressive environment (T regulatory cells, MDSCs) and on the tumor susceptibility to NK cytotoxicity.

Altogether, the combined treatment of CD1d and a low dose of cyclophosphamide may be useful to mount a powerful innate immune response against the tumor while retaining low systemic toxicity.

MAGE-A3 Antigen-specific Cancer Immunotherapeutic in Metastatic Melanoma: Final Results of a Randomized Open-label Phase II Study of the EORTC Melanoma Group (16032-18031)

*Brigitte Dreno**, L. Mortier†, C. Robert‡, V. Chiarion-Sileni§, A. Testori‖, M. Maio¶, A. Awada#, F. Lehmann**, W. Kruit††. **CHR Hotel Dieu, Nantes;* †*CHR, Lille;* ‡*IGR, Villejuif, France;* §*Azienda Ospedaliera, Padova;* ‖*Istituto Europeo di Oncologia, Milano;* ¶*Azienda Ospedaliera Universitaria Senese-Policlinico "Le Scotte", Siena, Italy;* #*Institut Jules Bordet, Brussels, Belgium;* ***GlaxoSmithKline Biologicals, Rixensart;* ††*Erasmus Medical Center, Rotterdam, Netherlands.*

Introduction: MAGE-A3 is a tumor-specific antigen expressed on multiple tumors but not on normal cells. It is expressed in 65% of metastatic melanomas. The MAGE-A3 antigen has been shown to be a target for antitumor $CD4^+$ and $CD8^+$ T cells. The recombinant MAGE-A3 protein has been combined with a potent immunostimulant, the subsequent product defines a new therapeutic class, the Antigen-specific Cancer Immunotherapeutics (ASCI).

Methods: A randomized open-label phase II study (NCT00086866) was designed to evaluate MAGE-A3 recombinant protein combined with 2 different immunostimulants (AS02B and AS15) as first-line metastatic treatment in MAGE-A3(+) cutaneous melanoma patients (unresectable or in transit stage III stage IV M1a). Primary end points were the rate of treatment-related NCIC-CTC grade 3 to 4 toxicity and the rate of objective response [complete response/partial response (PR)]. Secondary end points were immune response and other parameters of clinical activity. Identification of biomarkers associated with clinical response was achieved using gene expression profiling by microarrays on the tumor in patients who have taken prior treatment.

Results: At the time of the final analysis, median follow-up time was 25.3 months. Ninety-one of 165 screened patients had MAGE-A3(+) tumor biopsy, 75 were randomized and 72 patients were eligible. The safety profile was comparable in the 2 groups and almost all adverse events were grade 1 or 2. In the AS15 group, 3 patients were in complete response (11, 24+, 32+ mo) and 1 showed partial response (6 mo), whereas 1 patient in the AS02B group had a partial response (7 mo). Stable disease (for more than 16 wk) was seen in 5 patients in both groups. The overall survival was higher in the AS15 group: median value of 31.1 versus 19.2 months in the AS02B group. All patients developed anti – MAGE-A3 antibodies; the antibody titers were higher in the AS15 group. The anti – MAGE-A3 – specific $CD4^+$ T-cell stimulation was more frequent in the AS15 group and of higher amplitude.

Conclusions: Both investigated ASCI formulations showed acceptable safety results. The combination of recombinant MAGE-A3 with AS15 induced more objective clinical responses and elicited a stronger and more robust immune response. These results prompted the initiation of DERMA: the Phase III study currently enrolling 1300 patients with MAGE-A3(+) resected stage III cutaneous melanoma.

Tumor Endothelial Marker 1 as a Target for Genetic Vaccine

Andrea Facciabene, John Facciponte, George Coukos. *Ovarian Cancer Research Center, University of Pennsylvania, Philadelphia, PA.*

Tumor blood vessels are known to differ from vessels of normal organs and offer unique molecular targets for developing therapeutic interventions in cancer such as immunotherapy. There is a strong rationale for targeting tumor vasculature markers and stroma in addition to tumor. Endosialin (Tem1) has been claimed to be the most abundantly expressed tumor endothelial antigen, making it a prime candidate for vascular targeting purposes. Plasmid DNA vaccine is a safe and promising method for genetic vaccination and has been used against various pathogens and cancers. We constructed a plasmid DNA encoding the fully optimized sequence of mouse Tem1 fused with the minimized domain of tetanus toxin fragment C (Tem1-DOM). Immunization studies were carried out in C57BL/6 mice. Vectors encoding Tem1-DOM can break tolerance to Tem1 and elicits a significant immune cell-mediated response. In addition, vaccination based on the use of plasmid Tem1-DOM followed by electrogene transfer exerts a potent antitumor effect in the E6/E7 human papilloma virus-induced TC-1 model. Two different approaches were used to investigate the effects of Tem1-DOM on tumor growth, preventive and therapeutic. In the preventive approach, groups of 10 animals were immunized with 3 DNA immunizations in a weekly schedule and 1 week after the last immunization the animals were challenged with a lethal dose of TC-1. The vaccination with Tem1-DOM results in 100% protection from tumor growth. In the therapeutic approach, the animals were challenged with a lethal dose of TC-1 and 5 days later the animals were immunized as before. Tem1-DOM fusion eradicates the tumor in 5 of 10 animals and reduces tumor burden in the remaining animals. We determined that $CD8^+$ T cells were primarily responsible for Tem1-DOM immune-mediated effects. $CD4^+$ T cells also contributed to the anti-tumor response whereas serum did not contribute significantly. Sequentially, we were able to demonstrate an E7 cross-priming effect, showing a CD8-specific human papilloma virus E7 immune response in the mice challenged with TC-1 and immunized with Tem1-DOM. These results further corroborate the power of genetic vaccines, the efficacy of the tetanus toxin-derived protein in enhancing antitumor responses, and the unexpected powerful antitumor effects of a vaccination directed against tumor vasculature.

Novel Immunogenic HLA-A*0201-restricted Epidermal Growth Factor Receptor -specific T-cell Epitope in Head and Neck Cancer Patients

Pedro Andrade Filho, Steve C. Lee, Andres Lopez-Albaitero, *Robert L. Ferris. Cancer Immunology Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

Therapeutic targeting of the epidermal growth factor receptor (EGFR), which is highly overexpressed and correlated with poor prognosis in colorectal and head and neck squamous cell carcinoma (SCCHN) has shown clinical efficacy using the blocking monoclonal antibodies (mAbs), cetuximab, or panitumumab, but only in 10% to 20% of patients. Clinical responsiveness is correlated with certain Fcγ receptor (FcγR) genotypes, suggesting that immune activity may contribute to therapeutic efficacy. In addition, cetuximab-resistant tumor cells exhibit ubiquitination and degradation of EGFR, which would increase its processing as a tumor antigen for cytotoxic T lymphocyte (CTL) lysis. Thus, T-cell – based immunotherapy might enhance the antitumor efficacy of EGFR-specific mAbs, but CTL epitopes are poorly defined. To permit combinatorial EGFR-targeted immunotherapy, we identified a novel immunogenic wild-type sequence peptide, EGFR853-861, and modified its anchor sequence to enhance HLA-A*0201 binding and stimulation of cross-reactive anti-wt EGFR853-861 – specific CTL. Cross-reactivity was also observed with HER2861-869. EGFR853-861 – specific CTL recognition of SCCHN cells was increased by the incubation of tumor cells with cetuximab, which led to EGFR degradation. In addition, EGFR853-861 – specific CTLs were elevated in the circulation of SCCHN patients compared with healthy control peripheral blood mononuclear cells. Thus, a novel, immunogenic EGFR-encoded CTL epitope may be incorporated into vaccines and would be useful for combinatorial immunotherapy with EGFR-specific mAbs in cancer patients.

Defining the Role of TKIS in Reducing Immune Suppression While Improving T-cell Responsiveness and Efficacy of Immunotherapy in the Treatment of Tumors

*James H. Finke**, Jennifer S. Ko*†, Brian Rini‡, Patricia Rayman*, Joanna Ireland*, Walter Storkus§, Anamika Bose§, Soumika Biswas*, Kaushik Biswas*, Peter Cohen‖. **Immunology, Cleveland Clinic Foundation; †Pathology, Case Western Reserve University; ‡Solid Tumor Oncology, Cleveland Clinic Taussig Cancer Institute, Cleveland, OH; §Dermatology & Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA; ‖Mayo Clinic, Scottsdale/Phoenix, AZ.*

Immunotherapy for the treatment of metastatic renal cell carcinoma (RCC) has shown a 15% to 20% response rate, whereas in a small minority of patients, treatment with interleukin-2 induced long-term survival. The relatively weak response to immunotherapy by the majority of patients is likely related to tumor-induced suppression of T-cell immunity. Indeed, peripheral blood T cells from RCC patients display a diminished capacity to generate a type-1 T-cell interferon (IFN)-γ response, which is considered critical for an antitumor immune response. Recently, the small molecule receptor tyrosine kinase inhibitor, sunitinib, which targets several receptors in the vascular endothelial growth factor receptor family, has become first-line treatment for patients with mRCC. This drug has increased the response rate over that observed with cytokine therapy and has been relatively successful at extending patient survival although it is not curative. We have shown that treatment of RCC patients with sunitinib reverses T-cell suppression as shown by the increased T-cell production of IFN-γ along with increased frequency of RCC antigen-specific (EphA2 and MAGE6 peptides) T cells. The improvement in T-cell response coincided with a dramatic reduction in the number of myeloid-derived suppressor cells (MDSC) and to a lesser degree in T regulatory cell numbers. Parallel to the human findings, we and other researchers show that sunitinib inhibits MDSC accumulation and improves T-cell function (enhanced proliferation and IFN-γ production) in mice bearing several tumor types (4T1, RENCA, CT26, and M05B16.OVA, 40 mg/kg). Similar to the in vivo treatment with sunitinib, in vitro MDSC depletion using either anti – Gr1-magnetic beads or anti-CD15 beads restored T-cell function within murine splenocytes or peripheral blood mononuclear cells of RCC patients, respectively. The mechanisms by which sunitinib inhibits accumulation of MDSC in a tumor-bearing host is under investigation and recent findings suggest that sunitinib may significantly block the proliferation of the monocytic MDSC subset while possibly promoting apoptosis of the neutrophilic MDSC subset (n-MDSC), the latter of which is the most prevalent subset in most mouse models and RCC patients. Additional studies show that tumor-conditioned media (TCM) from RCC lines can not only induce n-MDSC in the whole blood of a healthy donor but can also extend their survival in vitro. Although the treatment of whole blood with sunitinib in the presence of TCM was unable to prevent the induction of MDSC by TCM, TCM derived from sunitinib (0.1 μg/mL)-treated tumor cells was less able to activate n-MDSC. Additional experiments illustrate that sunitinib can enhance the efficacy of 2 different immunotherapy approaches in mouse tumor models. Treatment of Balb/c mice bearing established CT26 tumors showed that although intratumoral CpG (ODN1826, 50 μg) and/or pIC (40 μg), and/or intraperitoneal sunitinib (40 mg/kg) had minimal effectiveness, the combination of CpG, pIC, and sunitinib significantly prolonged survival. This therapeutic effect persisted until treatment was stopped. We observed superior antitumor efficacy in mice with established M05 (B16.OVA) melanoma after treatment with sunitinib plus DC/OVA peptide-based vaccination that was paralleled by the greatest degree of specific T-cell priming and $CD8^+$ T-cell infiltrate. These data support combinational therapies implementing both sunitinib and cancer-specific vaccination.

Self-adjuvanting Messenger RNA-based Vaccines Induce Complete Adaptive Immune Responses

Mariola Fotin-Mleczek, Katharina M. Duchardt, Jochen Probst. *CureVac GmbH, Tübingen, Germany.*

Direct vaccination with messenger RNA (mRNA) molecules, encoding tumor-associated antigens is a novel and promising approach in the field of cancer immunotherapy. An important advantage of using mRNA for the purpose of vaccination is that the same molecule can not only provide an antigen source but at the same time can bind to pattern recognition receptors, thus stimulating innate as well as adaptive immunity. The combination of 2 activities within the same molecule has the advantage of targeting the same cell population. It ensures that antigen-presenting cells are not only loaded with antigen, which is expressed and presented on their cell surface, but simultaneously activated by adjuvant. However, achieving both immune stimulation and efficient protein translation remains challenging, as the formulation of mRNA, which is required to support its immune stimulatory activity, may inhibit its translational activity. In this study, a particular formulation of mRNA with the cationic protein protamine (PRT-RNA) was developed to support both activities. We show that the formulated mRNA encoding Photinus pyralis luciferase, supports protein translation in mice after intradermal injection and stimulates cytokine production (tumor necrosis factor-α, interleukin-6, interferon-α) in human peripheral blood mononuclear cells in vitro. Importantly, we show that intradermal injection of formulated mRNA encoding Gallus gallus ovalbumin leads to the induction of adaptive immune responses comprising induction of antigen-specific humoral responses as well as activation of antigen-specific $CD4^+$ and $CD8^+$ T cells and memory T cells. Prophylactic as well as therapeutic vaccination of mice with formulated mRNA mediates a strong antitumor response. The immunogenicity of formulated mRNA is not restricted to model antigen but also exists for tumor-associated antigens, and can be shown for human prostate-specific membrane antigen. Moreover, (in our hands) tumor vaccines developed on the basis of protamine-formulated mRNA exhibit higher potency compared with other antigenic formats such as peptide or recombinant protein. In conclusion, protamine-formulated mRNA with self-adjuvanting activity induces complete adaptive immune responses and mediates sustained antitumor activity. Thus, our results are highly relevant for the development of effective mRNA-based vaccines. A phase I clinical trial in hormone refractory prostate cancer patients using PRT-RNA is currently underway.

Identification of Immunogenic Major Histocompatibility Complex Class I and Class II Epitopes From the Tumor Rejection Antigen PAX2

*Doreen Haase**, Anne Marie Asemissen†, Stefan Stevanovic‡, Anne Letsch†, Antonia Busse†, Eckhard Thiel†, Hans-Georg Rammensee‡, Ulrich Keilholz†, Carmen Scheibenbogen*. **Institute of Med. Immunologie, Charite-CCM; †Hämatologie/Onkologie, Charite-CBF, Berlin; ‡Institute of Zellbiologie-Immunologie, University of Tübingen, Tübingen, Germany.*

PAX2, a member of the highly conserved embryonic transcription factor family of paired box genes (PAX), is aberrantly expressed in a variety of solid and hematologic malignancies. PAX2 regulates the transcription factor, WT1, which is a promising target of cancer immunotherapy. We applied a modified reverse immunology strategy to identify immunogenic epitopes of PAX2, which could be useful for cancer immunotherapy. Thirteen HLA-A0201-restricted major histocompatibility complex class I candidate epitopes were predicted with its binding algorithm (*www.syfpeithi.de*) and a proteasome cleavage algorithm (*www.paproc.de*) and screened for recognition by T cells from HLA-A*02-positive cancer patients using intracellular cytokine cytometry. Nine of 20 colon carcinoma patients, 1 of 13 renal cell carcinoma (RCC) patients, and 2 of 17 lymphoma patients showed a spontaneous CD8 T-cell response toward at least 1 of 6 PAX2 candidate epitope pools. None of the 20 healthy volunteers presented reactivity toward any of the PAX2 peptides. Cytotoxic T cells specific for the PAX2.337-345 (TLPGYPPHV) epitope could be generated repeatedly from 1 RCC patient after the depletion of $CD4^+CD25^+$ regulatory T cells

and lysis of PAX2-positive tumor cells was demonstrated. From the region against which we had most frequently observed CD8 T-cell responses, we selected a 21-mer peptide, PAX2.157-177, to screen for CD4 T-cell responses. Of the 6 patients screened so far, 2 RCC patients had a CD4 and CD8 T-cell response against PAX2.157-177 with 1 of them showing a high frequency of interleukin-2 – producing CD8 T cells (2.75%, background subtracted) and tumor necrosis factor-producing CD4 T cells (0.19%, background subtracted). We were able to identify PAX2 as a target of CD8 and CD4 T-cell responses in cancer patients. Thus, PAX2 is another embryonic transcription factor, which is of potential interest as an immunotherapy target antigen.

A High-throughput Screening Assay to Identify Agents That Enhance T-cell Recognition of Human Melanomas

*Timothy J. Haggerty**†, Estelle Newton*, Ian S. Dunn*, Lenora B. Rose*, James T. Kurnick*†. **CytoCure, Beverly;* †*Pathology, Massachusetts General Hospital, Boston, MA.*

To identify novel agents enhancing antigen expression on human melanomas and thereby increasing T-cell – mediated recognition of tumor cells, we devised a high-throughput screening (HTS) process to test libraries of biologically available drugs that could improve the immunotherapy of melanoma. This assay uses Jurkat T cells transduced with a lentiviral vector system for the expression of a T-cell receptor (TCR) specific for a Melan-A/MART-1 peptide restricted by human leukocyte antigen-A2. When these TCR-transduced T cells recognize their cognate antigen on antigen-presenting tumors, they produce interleukin (IL)-2 that can be readily detected in a quantitative enzyme-linked immunosorbent assay assay. Moreover, this effect was initially shown to entirely conform to the predicted peptide and human leukocyte antigen recognition specificity. As proof –of principle, we screened a library of 480 compounds (with known biologic activity) for the ability to induce enhanced antigen expression on a melanoma cell line. We have previously described interferon-β as an agent that can increase antigen expression in melanomas and serve as an internal positive control. The assay showed low variability, good reproducibility, and excellent Z scores. Seven positive hit compounds inducing IL-2 production were identified from the library (1.5%). PMA, a known inducer of T-cell activation, was found among these, but this drug did not directly promote tumor antigen expression. In contrast, 6 of the 7 hits elicited positive assay signals through the augmentation of tumor antigen expression. Another class of agents is exemplified by daunorubicin, which we have previously shown to enhance antigen expression in melanoma cells. In our assay, daunorubicin interferes with T-cell activation and consequently abrogates their IL-2 production. This demonstrates that some agents may enhance antigen expression, yet be therapeutically compromised by the suppression of immune effector cells. The assay we have devised therefore allows screening of large numbers of compounds with potential benefit to immunotherapy, with simultaneous rejection of agents that depress immunity. Using robotics, this assay should be readily adaptable to large-scale HTS of extensive compound libraries. In summary, we have implemented an HTS-compatible assay system allowing us to identify several compounds not hitherto known to enhance antigen expression in melanomas. This system should serve as a practical model for the identification and development of novel drugs to improve T-cell recognition of tumors, and the consequent enhancement of immunotherapeutic outcomes.

Peptide/Incomplete Freund Adjuvant Emulsion Depots are a Graveyard for Tumor Antigen-specific $CD8^+$ T Cells

Willem Overwijk, *Yared Hailemichael, Zhimin Dai, Nina Jaffarzad, Patrick Hwu. Melanoma Medical Oncology, MD Anderson Cancer Center, Houston, TX.*

Defined peptides are attractive cancer vaccine candidates that have shown some success in clinical trials. Peptide vaccines are commonly administered as water-in-oil emulsions in incomplete Freund adjuvants (IFAs). We studied the immune response of gp100-specific transgenic pmel-1 $CD8^+$ T cells and endogenous, OVA -specific $CD8^+$ T cells to peptide/IFA emulsion vaccination. We found that although T cells reliably proliferated and acquired an effector function, their lifespan was brief and secondary response to boosting was poor. In addition, virus-induced primary and secondary T-cell responses were abrogated by concurrent peptide/IFA vaccination. This vaccination-induced tolerance was dominant and independent of regulatory T cells. The tolerizing activity of the peptide/IFA vaccine persisted for more than 30 days and correlated with continuous peptide presentation by B cells and dendritic cells in the vaccine-draining lymph node, although B cells were not required for the induction of tolerance. Trafficking of bioluminescent T cells showed dramatic and near-absolute sequestration of activated, antigen-specific T cells at antigen-containing vaccine depots but not control vaccine depots or antigen-positive tumors. We propose that long-lived IFA vaccine depots function as a sink and graveyard for vaccination-induced, tumor-specific T cells.

Enhancement of a P53-based Cancer Vaccine by Heterologous Prime/Boost Immunization and Toll-like Receptor Stimulation

*Hidenobu Ishizaki**, Guang-Yun Song*, Vafa Shahabi‡, Don Diamond†, Joshua Ellenhorn*. **Department of General and Oncologic Surgery, City of Hope National Medical Center;* †*Division of Translational Vaccine Research, Beckman Research Institute of the City of Hope, Duarte, CA;* ‡*Research and Development, Advaxis Inc, North Brunswick, NJ.*

The p53 gene product is overexpressed by almost 50% of cancers, making it an ideal target for cancer immunotherapy. We previously showed that a modified vaccinia virus Ankara (MVA) vaccine expressing human p53 (MVAp53) could overcome tolerance to p53 in human p53 knock in the Hupki mice model. One approach to stimulating strong tumor-specific immunity involves repeated the vaccination with a recombinant vaccine vector, such as MVAp53. However, boosting immune responses by repeated administration (homologous boosting) of a recombinant vaccine virus can be inefficient, because of the development of neutralizing immunity to the vaccine viral vector backbone, which interferes with the expression of the tumor associate antigen. To address this issue, we examined the role of 2 different vaccine vectors provided in sequence as a heterologous prime/boost immunization. We employed attenuated recombinant *Listeria monocytogenes* expressing human p53 in addition to MVAp53. Heterologous prime/boost immunization resulted in a significant increase in p53-specific $CD8^+$ and $CD4^+$ T cells compared with homologous prime/boost immunization using a single vector. Hupki mice were evaluated as a tolerant model to explore the capacity of heterologous prime/boost immunization to overcome tolerance and reject human p53-expressing tumors. Heterologous prime/boost immunization induced protection against tumor growth but had only modest effects on established tumors in this model. To enhance the immune response, we used synthetic double-strand RNA [poly (I:C)] and unmethylated CpG sequences (CpG-ODN) to activate the innate immune system via toll-like receptors. Treatment of established tumor-bearing Hupki mice with poly (I:C) and CpG-ODN in combination with heterologous prime/boost immunization resulted in potent antitumor effects relative to treatments with either agent alone. These results suggest that heterologous prime/boost immunization and toll-like receptor stimulation may be useful in increasing the efficacy of the recombinant MVAp53 cancer vaccine.

Impact of Radiation on Immunotherapy Targets

Jennifer C. Jones, Susan J. Knox. *Radiation Oncology, Stanford University, Stanford, CA.*

Radiation therapy (RT) is widely used to palliate metastatic disease. Optimal RT + immunotherapy (IT) combinations may promote long-term immune responses against unirradiated tumor

sites. To design effective RT + IT combination therapies, we determined the effects of RT on (1) the survival of critical immune cell subsets and (2) the expression of key costimulatory/coinhibitory molecules.

Materials and Methods: Single, 5 mm, A20 lymphoma and 4T1 mammary carcinoma tumors were established in BALB/c mice. RT (0, 2, and 20 Gy; 200 kV orthovoltage) was delivered in a customized jig. Dose-response curves were determined for cells from the spleen and primary tumor at 24, 48, and 72 hours. Late effects were examined 6 and 10 days after RT. Cell subsets assessed by flow cytometry included T regulatory ($CD4^+$, $CD25^+$), natural killer (NK), NKT (DX5 +, TCR +), MSC (CD11b +, Gr-1 +), M1 and M2 macrophages, and CD11c dendritic cells. With 8-color flow cytometry, expression of costimulatory and coinhibitory molecules on each of these cell subsets was assessed simultaneously.

Results: RT significantly enriches the relative proportions of NKT cells, regulatory T cells, and myelo-monocytic cell subsets. In the tumor microenvironment, A20 cells are more sensitive to radiation-induced apoptosis than 4T1 cells, and the changes in the tumor-infiltrating immune cells are also distinct. RT selectively induces the expression of certain receptors, such as CD40, 4-1BB, PD-1, and PD-L1. Levels of PD-1 and its ligand, PD-L1, increase in a dose-dependent manner within 48 hours, followed by a gradual decline 4 to 6 days after RT. The identification of PD-1 and PD-L1 as RT-induced coinhibitory receptors is novel and of particular therapeutic interest. We also find that the expression of other cell deaths or stress-associated receptors, such as CD40 and 4-1BB, is increased after RT.

Conclusions: In a survey of the effects of RT on the cells and costimulatory/coinhibitory receptors that regulate tumor immunity within the tumor microenvironment, we identified PD-1, CD40, and 4-1BB (and their respective ligands) as promising receptor targets for RT + IT therapies. We also identified immune cells most likely to be present in the irradiated site during the 24 to 48 hours when immune reprogramming may occur. Specifically, we find that the antigen-presenting cell subsets (CD11b monocyte/macrophage populations) and certain T-cell subsets (NKT and regulatory T cells) are relatively radioresistant among the immune cells in tumors. Therefore, IT strategies that promote effective CD11b activation, enhance NK T-cell responses, or inhibit the locally immunosuppressive effects of regulatory T cells may be especially advantageous for use in conjunction with RT. Similarly, therapeutic blockade of the inhibitory effects of PD-1 (and PD-L1) may be a successful strategy for RT + IT combination therapy.

Characterization of Tumor-infiltrating Myeloid Cells in a Human Leukocyte Antigen-DR2B Transgenic Mouse Model of Prostate Cancer

*Elena N. Klyushnenkova**†, Justin Sausville*, Richard B. Alexander*†. **Department of Surgery, University of Maryland School of Medicine; † VA Maryland Health Care System, Baltimore, MD.*

We have recently described a novel mouse model of prostate cancer in transgenic mice engineered to express human leukocyte antigen (HLA)-DRB1*1501 (DR2b in old nomenclature) (*J Immunol.* 2009;182:1242–1246). We found that TRAMP tumor cells engineered to express prostate-specific antigen (PSA) were frequently rejected by HLA-DR2b − F1 mice but grew in HLA-DR2b + F1 littermates. CD8 T cells secreting interferon-γ in response to TRAMP-PSA tumor cells as well as the $H\text{-}2D^b$-restricted immunodominant peptide PSA (65-73) were significantly more frequent in splenocytes from HLA-DR2b − F1 mice inoculated with TRAMP-PSA tumors compared with HLA-DR2b + tumor-bearing F1 littermates. In contrast to CD8 T-cell responses, antibody responses to PSA were strong in HLA-DR2b + F1 mice bearing TRAMP-PSA tumors and were virtually undetectable in HLA-DR2b − F1 littermates The lack of the antibody response in HLA-DR2b − F1 mice was most likely due to the lack of strong $I\text{-}A^b$-restricted CD4 T-cell response, whereas HLA-DRB1*1501 (DR2b) allele was "permissive" for PSA-specific responses. These data suggested that the presence of the HLA-DR2b transgene in the mice somehow led to the inhibition of the cytotoxic T-cell response to PSA expressed by the tumor cells. We also analyzed the composition of the tumor-infiltrating myeloid cells in HLA-DR2b + and HLA-DR2b − F1 mice bearing TRAMP-PSA tumors. Matrigel implants impregnated with tumor cells were harvested 2 weeks after tumor inoculation, enzymatically digested, and leukocytes were purified by discontinuous Percoll gradient centrifugation and analyzed by flow cytometry. The analysis showed a profound increase in the number of tumor-infiltrating myeloid cells in TRAMP-PSA tumor implants derived from HLA-DR2b + F1 mice compared with HLA-DR2b − F1 mice. More detailed phenotypic analysis of the $CD11b^+$ population showed that it consisted of 3 subpopulations: Ly6G + Ly6C − PMN cells and Ly6G-Ly6C + and Ly6G-Ly6C − monocytes. Ly6C + monocytes are considered to be precursors for macrophages and dendritic cells in inflammatory conditions, whereas Ly6C − monocytes may represent steady-state precursor cells for tissue macrophages. The phenotypic and functional properties of these subpopulations and their role in tumor progression in our model are under investigation.

Supported by a grant from the US Department of Veterans Affairs.

Helper Activity of Natural Killer Cells During the Dendritic Cell-mediated Induction of Melanoma-specific Cytotoxic T Lymphocyte Responses

*Robbie B. Mailliard**, Stergious Moschios†‡, Howard Edington†‡, Michael T. Lotze*‡, John Kirkwood†‡, Pawel Kalinski*‡. **Departments of Surgery; †Medicine, University of Pittsburgh; ‡University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

In addition to their cytotoxic effector functions, natural killer (NK) cells are also capable of "helper" activity, inducing dendritic cell (DC) maturation but preventing the maturation-related DC "exhaustion," resulting in mature DCs with enhanced ability to produce interleukin (IL)-12 upon subsequent contact with T cells (type-1 polarized DC; DC1). Here, we show that the NK cell-mediated type-1 polarization of DCs can be applied in clinically relevant serum-free cultures to enhance the efficacy of DCs in inducing melanoma-specific cytotoxic T lymphocytes. NK cells isolated from melanoma patients responded to several defined combinations of stimulatory agents, such as interferon (IFN)-α plus IL-18, IFN-α plus K562 cells (NK cell-sensitive leukemia), or IFN-α plus opsonized melanoma cells (nominally NK cell non-sensitive), with IFN-γ production and induction of type-1 polarized DC. Such NK cell-induced NKDC1 show a strongly enhanced (average of 64-fold) capacity to produce IL-12p70 on subsequent interaction with T cells, compared with nonpolarized tumor necrosis factor-α/IL-1β/IL-6/prostaglandin E_2-matured "standard" DC. Although NKDC1 expressed similar levels of CD86, CD83, and CD40, they expressed lower levels CCR7, which could be compensated by additional costimulation with poly-I:C. When compared in vitro with nonpolarized standard DC, NKDC1s were superior (average of 60-fold) in inducing high numbers of functional melanoma-specific cytotoxic T lymphocytes recognizing multiple melanoma-associated antigens and capable of killing melanoma cells. These results indicate that the helper function of NK cells can be used in clinical settings to improve the effectiveness of DC-based cancer vaccines.

Topical Peptide Immunization With NY-ESO-1B Peptide (SLLMWITQC) Using Resiquimod as an Immune Adjuvant

Aaron Mansfield, Lisa Kottschade, Rob McWilliams, Edward Creagan, Judith Kaur, Gary Croghan, Fernando Quevedo, Svetomir Markovic. *Department of Oncology, Mayo Clinic, Rochester, MN.*

Melanoma vaccines have not shown significant clinical benefit to date. Different immune adjuvants have been tested as part of melanoma vaccines to enhance their effectiveness. Resiquimod is a toll-like receptor (TLR) agonist that stimulates TLR-7 and TLR-8, and may therefore enhance vaccine immunogenicity. We tested topical administration of resiquimod as an immune adjuvant for a melanoma-associated tumor antigen peptide (NY-ESO-1b) when the latter was administered either subcutaneously or topically. A phase I clinical trial was designed to assess the safety and immunization efficacy of a resiquimod/NY-ESO-1b melanoma vaccine administered at different resiquimod concentrations through different routes of administration in HLA-A2+ patients with a history of resected melanoma. NY-ESO-1b was applied either subcutaneously or topically to the prepared skin. Immediately before the application of the topical vaccine, the volar aspect of the upper extremity was prepared using adhesive tape to promote mild irritation. Treatment cohorts varied by concentration of applied resiquimod and surface area of vaccine application. NY-ESO-1b dosage remained the same in all cohorts. At registration, patients were assigned to the currently open regimen of topical resiquimod application area and concentration to be administered in combination with 100 μg of NY-ESO-1B peptide vaccine. The concentrations of resiquimod under investigation were 0.01%, 0.06%, 0.1%, and 0.2%, and the surface areas of resiquimod application were 5 cm × 5 cm, 5 cm × 10 cm, 5 cm × 15 cm, and 5 cm × 20 cm. An immune response in patients with detectable pretreatment NY-ESO-1b tetramer levels was defined as at least a 2-fold increase in NY-ESO-1b tetramer levels ($CD8^+$/tetramer positive lymphocytes) in the 8 weeks after immunization. An immune response in patients with undetectable pretreatment NY-ESO-1b tetramer levels was defined as a detectable NY-ESO-1b tetramer level $\geq 0.1\%$ ($CD8^+$/tetramer positive lymphocytes) in the 8 weeks after immunization. Thirty patients were enrolled onto this study. No patient developed dose-limiting toxicity. The frequency of $CD8^+$ T-cell responses to NY-ESO-1b was 37% (11/30), which is in the range of responses seen in other vaccine trials using NY-ESO-1. The average magnitude of $CD8^+$ T cells to NY-ESO-1b in patients with measurable responses was 0.08% (0.04%-0.23%). The majority of patients with a larger surface area of application ($> 25\,cm^2$) showed a measurable tetramer response ($P = 0.13$ by χ^2). There was also a slight increase in measurable anti–NY-ESO-1 antibodies in treated patients. In conclusion, resiquimod is a safe and potentially useful immune adjuvant for topical peptide immunization with NY-ESO-1b.

A Novel Breast/Ovarian Cancer Peptide Vaccine Platform That Promotes Specific Type-1 but not Treg/TR1-type Response

*Karkada Mohan**†, Genevieve M. Weir*, Tara Quinton*, Leeladhar Sammatur*, Lisa D. MacDonald*, Alecia Grant*, Robert Liwski‡, Ridas Juskevicius‡, Gomathinayagam Sinnathamby§, Ramila Philip§, Marc Mansour*. **ImmunoVaccine Technologies; †Departments of Microbiology and Immunology; ‡Hematopathology, Dalhousie University, Halifax, NS, Canada; §Immunotope Inc, Doylestown, PA.*

In light of the relatively poor efficacy associated with current peptide-based cancer vaccines, a novel vaccine platform called DepoVax™ as a therapeutic vaccine for breast/ovarian cancer was developed. This water-free depot vaccine formulation (DPX-0907) has high immune-activating potential. A preclinical study was designed to examine the efficacy of this novel platform over conventional emulsion vaccine using human class I major histocompatibility complex transgenic mice. Naturally processed peptides bound to HLA-A2 molecules isolated from independent breast and ovarian tumor cell lines were isolated and used as antigens in DPX-0907 along with a proprietary adjuvant and a T helper peptide epitope. Efficacy of vaccine was tested in immunized HLA-A*0201/H2Dd transgenic mice by measuring the frequency of interferon (IFN)-γ – secreting cells in the draining lymph nodes, and regulatory T-cell frequencies in the spleen. Compared with a water-in-oil emulsion vaccine, DPX-0907 enhanced IFN-γ + $CD8^+$ T cells in vaccine site-draining lymph nodes, as seen by flow cytometry. This vaccine also increased the frequency of IFN-γ + cells in the lymph node as detected by a modified dendritic cell-based enzyme-linked immunosorbent spot assay. Notably, although conventional vaccine formulations elicited elevated levels of $CD4^+$Foxp3+ and IL10-secreting CD4 T cells in the spleen, this was not the case for DPX-0907 – based vaccines, with treated animals exhibiting normal levels of regulatory T cells. These data support the unique immune-stimulating capabilities of DPX-0907 tumor peptide vaccine formulation to elicit type-1 – dominated specific immunity. This vaccine might represent a potent therapeutic modality in the clinic for patients with breast/ovarian carcinoma. Moreover, this platform may also help in developing novel effective vaccines against other types of human cancers and infectious diseases.

DNA Vaccine Targeting Interleukin-13 Receptor α2 Induces Tumor Immunity in Murine Solid Tumor Models

Hideyuki Nakashima, Toshio Fujisawa, Syed R. Husain, Raj K. Puri. *Center for Biologics Evaluation and Research, FDA, Bethesda, MD.*

Interleukin-13 receptor α2 (IL-13Rα2) is a primary high-affinity binding and internalization subunit of IL-13R. Although high levels of IL-13Rα2 are expressed on a variety of human tumors, its precise role in tumor immunology is still not known. We showed earlier that IL-13Rα2 is a novel tumor antigen. In this study, we have developed murine models of tumors naturally expressing IL-13Rα2 (MCA304 sarcoma and 4T1 breast carcinoma) in syngeneic mice and examined the antitumor activity of IL-13Rα2 DNA vaccine with or without the extracellular domain of the IL-13Rα2 (ECDα2) protein mixed with CpG and IFA adjuvants as a boost vaccine. Three intramuscular prophylactic immunizations at 6, 4, and 2 weeks with DNA vaccine resulted in partial protection against MCA304 tumor challenge compared with the mock vector vaccine. A 64% reduction in tumor volume was observed compared with control on day 24. Two additional weekly (days 7 and 14 posttumor challenge) boost vaccinations with the ECDα2 protein further decreased tumor burden by 89% compared with the mock vector vaccine. In an established MCA304 tumor model, vaccination with IL-13Rα2 DNA on days 4, 9, 14, and 19 decreased the tumor burden by 79% on day 21 compared with the control receiving the mock DNA vaccine. Two additional ECDα2 boost injections on days 24 and 29 further reduced the tumor size. Similar results were also observed in a murine breast carcinoma model. IL-13Rα2 DNA vaccine-primed and ECDα2-boosted mice showed higher cytotoxic T lymphocyte activity and interferon-γ release from splenocytes in vitro compared with the controls in both tumor models. Immunohistochemical analysis showed the infiltration of CD4-positive and CD8-positive T cells and interferon-γ – induced chemokine (CXCL9 and CXCL10) expression in MCA304 tumor tissues of immunized mice. These results suggest that immunization with IL-13Rα2 DNA vaccine followed by ECDα2 boost inhibits tumor growth in a T-cell – dependent manner. Thus IL-13Rα2 is a potent target for cancer vaccine strategies and additional work is needed to analyze the therapeutic potential of this approach.

Type-1 Dendritic Cell Vaccines in Combination With Poly-ICLC-association Between Positive Tetramer Response and 6-month Progression-free Survival

*Hideho Okada**, Frank S. Lieberman*, Aki Hoji*, Pawel Kalinski*, Arlan H. Mintz*, David L. Bartlett*, Theresa L. Whiteside*, Lisa H. Butterfield*, Ronald L. Hamilton*, Andres M. Salazar†, Ian F. Pollack*. **University of Pittsburgh Cancer Institute, Pittsburgh, PA; †Oncovir, Inc, Washington, DC.*

Our previous preclinical studies have shown that intramuscular administration of a toll-like receptor 3-ligand poly-ICLC remarkably enhances the induction of type-1 cytotoxic T lymphocytes and improves the therapeutic efficacy of vaccinations against glioma-associated antigen-derived $CD8^+$ T-cell epitopes. We have developed a phase I/II trial. Human leukocyte antigen-A2+ participants with recurrent malignant glioma received intralymph node injections of type-1 dendritic cells loaded with human leukocyte antigen-A2 binding glioma-associated antigen peptides EphA2 (883-891), interleukin-13R α2 (345-353:1A9 V), YKL-40 (202-211), and GP100 (209-217: 2M) at 2-week intervals. Participants also received twice weekly IM injections of 20 μg/kg poly-ICLC. Participants who showed a positive radiologic response or stable disease without major adverse events were allowed to receive booster vaccines. Primary end points were the assessments of safety and immunologic responses. Clinical and radiologic responses were also evaluated. To date, 15 participants [8 with glioblastoma multiforme (GBM), 5 with anaplastic astrocytoma (AA), and 2 anaplastic oligodendroglioma (AO)] have received at least 4 vaccinations with no major adverse events. Increased $CD8^+$ cells reactive to EphA2 or interleukin-13R á2 tetramers were detected in postvaccine peripheral blood mononuclear cells in 9 of the 11 participants evaluated. These patients also showed upregulation of a chemokine receptor CXCR3 on $CD8^+$ peripheral blood mononuclear cells after vaccine administration, indicating that the vaccine regimen induced type-1 cytotoxic T lymphocyte responses. Five patients were progression free at 6 months (2 GBM, 2AA, and 1 AO). Among these, 4 patients are currently progression free at 17 (AA), 15 (GBM), 14 (AA), or 9 (AO) months after the first vaccine, and are receiving booster vaccines every 3 months. Although the trial enrolled mixed tumor types, Fisher exact test indicated an association between positive tetramer response and 6-month progression-free survival, suggesting a possible correlation between antigen-specific responses and clinical response. These interim data show preliminary safety, immunologic, and clinical activity of poly-ICLC – assisted type-1 dendritic cell-based vaccines.

Autologous Vaccine AHICE, Active Immune Therapy Against Breast Cancer

*Ioulia Papapolychroniadou**, Hannes Anthopoulos†, Konstantinos Papapolychroniadis‡, Vasilis Papadopoulos‡, Panagiotis Ginopoulos§, Epaminontas Fahantidis‡. **Department of Health and Human Sciences, Pharmaceutical Sciences, London Metropolitan University, London, United Kingdom; † K-Bio Clinical Biochemical Institute for Cell Biotechnology and Immunology, Munich, Germany; ‡1st Surgical Propedeutic Clinic, Aristotle University of Thessaloniki, Thessaloniki; §Department of Medical Oncology, St. Andrews Hospital, Patra, Greece.*

The autologous vaccine AHICE, active immune cancer therapy, which is directed and controlled over the complex sum of the immune-response products, is of high selectivity and specificity against tumor and its metastatic cells. In our clinical study, we report the results and conclusions after a combined unit study protocol of a series of 25 breast cancer patients in a period from February 2005 to February 2008. All these patients received the individually prepared autologous vaccine, AHICE, immune therapy. The duration of each one of that long-term active vaccine AHICHE cycle was 3 months. Before beginning, at every 3 weeks and at the end of the therapy cycle, a disease research control study was performed by imaging and hematologic tests. Using the findings of computed tomography, magnetic resonance imaging, or positron emission tomography scan images, the hematologic estimations of T lymphocyte immune phenotyping, biochemical liver tests (C-reactive protein, GLDH, and CHE) as well as tumor markers, we could evaluate disease control. After the statistical analysis of these results, we can conclude that the AHICHE therapy is an effective, well tolerated, without complications immune therapy, which is opening new therapeutic options in breast cancer management.

IDO/IDO2 Inhibition: A New Strategy to Drive Immunotherapeutic Responses in Cancer by Reversing Tumoral Immune Tolerance

George C. Prendergast. Lankenau Institute for Medical Research, Wynnewood, PA.

Small molecule inhibitors of the tryptophan catabolic enzymes, IDO1 and IDO2 (indoleamine 2,3-dioxygenase-1/2), represent novel immunotherapeutic modalities to treat cancer by reversing immune tolerance. In collaboration with academic, government, and biopharmaceutic groups, my laboratory has promoted preclinical and clinical development of IDO inhibitors. With our discovery that the tumor suppressor gene, Bin1, supports immune surveillance by restricting IDO1, we went on to identify several classes of small molecule inhibitors of IDO1 and/or IDO2. In several preclinical models of cancer, these agents exert antitumor effects that cooperate with "immunogenic" chemotherapeutic drugs to elicit tumor regression. Cancer relevance has been furthered with recent evidence that IDO1-deficient animals are resistant to inflammatory carcinogenesis. Our preclinical studies provided an impetus to evaluate the D stereoisomer of the well-studied IDO inhibitor 1MT (1-methyl-tryptophan). Pharmacologic and toxicological analyses revealed D-1MT to be very stable, well distributed, > 90% free in blood, orally bioavailable, and essentially nontoxic in animals even at very high doses. 1MT has been widely studied as a racemic mixture of D and L stereoisomers, however, D-1MT was found to be largely responsible for antitumor potency and T-cell stimulatory activity. The basis for D-1MT activity is complex and not fully understood. Our study indicates that IDO2 is preferentially inhibited by D-1MT with the action of both IDO2 and D-1MT linked to IDO1 action. The biochemical requirements for IDO2 catalytic activity differ from IDO1 and we find that IDO2 is posttranscriptionally regulated by IDO1, pointing toward an IDO1 > IDO2 genetic pathway that may explain the reliance of D-1MT on IDO1. D-1MT has served as a clinical "lead" compound in phase I studies conducted at 2 US sites. Initial findings suggest that orally administered D-1MT can elicit biologic activity and radiologic responses at safely tolerated doses. Ongoing studies focus on mechanistic questions about the regulation, function, and role of IDO1 and IDO2 in cancer and clinical responses to D-1MT.

Acknowledgements: The author expresses gratitude for long-standing collaborations in this area with Alex Muller (Lankenau Institute for Medical Research), Andy Mellor and David Munn (Medical College of Georgia, GA), Bill Malachowski (Bryn Mawr College), and Rick Metz, Chuck Link, Mario Mautino, and Nick Vahanian (New Link Genetics Corporation). The author declares a conflict of interest due to personal financial interests, grant support, and a consultancy role with New Link Genetics Corporation, which is developing D-1MT and other IDO inhibitors to treat cancer and other diseases.

References:

1. Muller, et al. Inhibition of indoleamine 2,3-dioxygenase, a target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. *Nature Med*. 2005;11:312–319.
2. Hou, et al. Inhibition of IDO in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with anti-tumor responses. *Cancer Res*. 2007;67:792–801.
3. Metz, et al. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor IDO inhibitory compound D-1MT. *Cancer Res*. 2007;67:7082–7087.
4. Kumar, et al. Indoleamine 2,3-dioxygenase is the anticancer target for a novel series of potent naphthoquinone-based inhibitors. *J Med Chem*. 2008;51:1706–1718.
5. Muller, et al. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3-dioxygenase. *Proc Natl Acad Sci USA*. 2008; 105:17073–17078.
6. Katz, et al. Indoleamine 2,3-dioxygenase in T cell tolerance and tumoral immune escape. *Immunol Rev*. 2008;222: 206–221.

Synergistic Effects of Chemoimmunotherapy Combination: Mechanisms Involved and Implications for the Design of Therapeutic Protocols Against Cancer

Antonella Sistigu*, Mara Valentini*, Iole Macchia*, Laura Bracci*, Federica Moschella*, Eleonora Aricò*, Giovanna Schiavoni*, Francesca Urbani*, Imerio Capone*, Paola Nisticò†, Virginia Ferraresi†, *Enrico Proietti**. **Istituto Superiore di Sanità; † Regina Elena Cancer Institute, Rome, Italy.*

Although cancer chemotherapy has been usually considered as immunosuppressive, recent evidence, stemming from studies in animal models, has pointed out that, to induce a consistent antitumor response, immunotherapy needs to be combined with chemotherapy. Nowadays, cyclophosphamide (CTX) represents the gold standard chemotherapeutic agent able to potentiate the effectiveness of cancer immunotherapy. The underlying mechanisms include the reduction of regulatory T-cell number and the induction of a plethora of immunomodulatory cytokines (cytokine storm) that leads to homeostatic proliferation and tumor infiltration by T lymphocytes. To further dissect the mechanisms underlying the immunomodulatory activity of CTX, the global gene expression profiles induced by this drug were studied by microarray analysis. This analysis showed that CTX profoundly affects gene expression early after treatment inducing the upregulation of several genes involved in the control of the immune response, cell migration, and cell differentiation. Of note, a day after CTX administration, gene expression levels of the factors involved in a Th17 type of immune response, were simultaneously increased with respect to untreated mice. As the induction of an effective antitumor response requires the active participation of host antigen-presenting cells, we also investigated the effects of CTX treatment on dendritic cells (DCs) in vivo. We showed that in mice implanted with EG7.OVA thymoma, CTX treatment induced a transient reduction of total bone marrow cells, but not of DC precursors, which, instead, proliferated displaying enhanced DC generation capabilities in vitro. Accordingly, in secondary lymphoid organs, conventional $CD8\alpha^+$ DCs, the key DC subset specialized in the cross-presentation of cell-associated antigens, underwent a transient and selective depletion followed by a rebound phase. In addition, plasmacytoid DCs, the main type I interferon producers, progressively accumulated in the spleen and in the lymph nodes of CTX-treated tumor-bearing mice. Interestingly, the percentages of myeloid-derived suppressor cells dramatically decreased in tumor-bearing mice, early after CTX administration, and remained at low levels for up to 10 days after treatment. These findings helped to perform a "proof of concept" study in melanoma patients combining dacarbazine (an alkylating agent as well as CTX), and antitumor peptide vaccination.

Exploiting the Humoral Immune Response to Identify Tumor/Tumor-associated Antigens Recognized After Combination Immunotherapy in Men With Hormone-refractory Prostate Cancer

*Sachin Puri**, James A. Thompson*, Janet C. Siebert†, Tarsem Moudgil*, Theresa Ratzow*, Shawn Jensen*, Michael Lacelle*, Daniel Haley*, Christian H. Poehlein*, Edwin Walker*, Nathalie Sacks‡, Kristen Hege‡, Hong-Ming Hu*§, Todd Coffey*, Carlo Bifulco*, Walter J. Urba*, Brendan Curti*, Bernard A. Fox*‖. **Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center; §Department of Radiation Oncology; ‖Molecular Microbiology And Immunology, Oregon Health & Science University, Portland, OR; † CytoAnalytics, Denver, CO; ‡Cell Genesys Inc, South San Francisco, CA.*

Detecting a tumor-specific immune response after immunotherapy is complex and fraught with hurdles. Although it is particularly problematic when complex vaccines (allogeneic tumor cells) are administered, peptide or protein vaccines may also lead to epitope spreading against an unrelated tumor antigen. Given the spectrum of potential targets, how is it possible to study the immune response that develops? We hypothesize that the development of a strong T-cell response will also lead to the generation of a B-cell response against the same antigen. Therefore, the identification of a new antibody response after immunotherapy may provide a surrogate for the generation of a T-cell response. To begin to address this hypothesis, we used Invitrogen's ProtoArray (8217 human proteins spotted in duplicate) to assess the spectrum of antibodies before and at 11 weeks after the start of immunotherapy. The phase I/II clinical trial randomized patients to either (A) GVAX immunotherapy for prostate cancer (2 prostate cancer cell lines that secrete granulocyte macrophage-colony-stimulating factor, Cell Genesys Inc) only, (B) $350\,mg/m^2$ cyclophosphamide for 3 days followed by the reinfusion of autologous peripheral blood mononuclear cells and immunotherapy, and (C) the same treatment as cohort B with the addition of fludarabine ($20\,mg/m^2 \times 3\,d$). As analysis of prostate-specific antigen-doubling time (DT) showed, despite lymphodepletion, that men in cohort C had the greatest increase in prostate-specific antigen-DT ($P = 0.065$), we focused our analysis of antibody responses on the 4 patients in this cohort. There were at least 10 proteins recognized by 2 or more men in this cohort. Five of these were previously described prostate antigens; however, the remaining 5 have not been reported previously. To ascertain whether these responses are against antigens expressed by the patient's tumor, we are isolating circulating tumor cells from cryopreserved peripheral blood. Initial studies document that circulating tumor cells can be detected in some patients and the characterization of expression levels for the 10 genes is underway. A detailed discussion of the data analysis method is presented in another poster.

Supported by The Prostate Cancer Foundation, DAMD Grant PC02009, Robert W. Franz, and the Chiles Foundation.

The Melanoma Vaccine Microenvironment: Do Negative Contributors of Immune Function Compartmentalize the Vaccine Site?

Jochen Schaefer, Donna Deacon, Mark Smolkin, James Patterson, Gina Petroni, Craig Slingluff. *University of Virginia, Charlottesville, VA.*

Purpose: Melanoma vaccines may offer clinical benefit, but have not been optimized. An underinvestigated aspect of vaccine biology is at the vaccine site itself, an understanding of which is arguably valuable for targeting approaches to improve cancer vaccines. We hypothesized that a vaccine incorporating an incomplete Freund adjuvant would induce dermal accumulation of immune cells, including Th1 and Tc1 lymphocytes and mature dendritic cells (DCs) but that repeated vaccination may also induce regulatory processes.

Experimental Methods: Excisional biopsy specimens of vaccine sites were obtained at 5 time points (prevaccine, weeks 1, 3, 6, and 12) in a randomized pilot study of cellular and molecular events at the cutaneous site of 6 weekly multipeptide vaccines. Hematoxylin-eosin and immunohistochemical preparations of paraffin-embedded skin sections from 24 patients were examined in 3 cutaneous layers: papillary dermis, reticular dermis, and subcutis; positive cells were counted by a pathologist in 10 high-power fields per layer.

Results: $CD4^+$ and $CD8^+$ T cells increase in all the layers over time ($P < 0.001$). $CD20^+$ B cells increase to a lesser degree. Mature and immature DCs are most numerous superficially and decrease slightly with dermal descent ($P < 0.001$). No increase in mature ($CD83^+$) DCs is seen over time. Mature DCs ($CD83^+$) cluster around superficial dermal vessels in aggregates with lymphocytes; however, immature (CD1a+) DCs are more randomly distributed. Th2 (GATA3+) cells increase disproportionately and dominate Th1 (T-bet+) cells after 1 vaccine; however, Th1 cells become more dominant after 3 vaccines ($P < 0.001$). With repeated vaccines, FoxP3+ lymphocytes also accumulate at the vaccine site so that the CD8:FoxP3 ratio declines from a high of over 7:1 prevaccine to only 2:1 after 3 vaccines. Eosinophils accumulate dramatically after 3 vaccines; they are more numerous in deep dermis and subcutis than in superficial dermis ($P < 0.001$).

Conclusions: Cells putatively supporting a cytotoxic T-cell response accumulate mostly in superficial dermis, whereas eosinophils and putative regulatory T cells are abundant in deeper layers. Thus, deep dermis and subcutis may represent less favorable environments for the induction of Tc1 and Th1 responses. Temporal changes in Th1 and Th2 dominance in the vaccine microenvironment support repeated vaccinations in the same location; however, accumulation of cells with putative negative influence challenges the value of more than 3 repeat vaccinations. The vaccine site microenvironment may have critical negative regulatory features that could be targeted locally with new adjuvants or immune modulators to improve the ability to generate T-cell responses against melanoma antigens.

Enhancing Cancer Vaccines as Monotherapy and in Combination Therapies

Jeffrey Schlom. National Cancer Institute, NIH, Bethesda, MD.

We have taken the approach that vaccine efficacy can be enhanced by the use of poxviral vectors expressing transgenes encoding 1 or more tumor-associated antigens and 3 T-cell costimulatory molecules (B7.1, ICAM-1, LFA-3, designated as TRICOM). A recent 43-center randomized, phase II, vector-controlled study in patients with metastatic castrate-resistant prostate cancer demonstrated that the prostate-specific antigen-TRICOM vaccine increased patient survival versus vector ($P = 0.006$). A concurrent phase II study showed similar findings and provided evidence of immunologic and patient characteristics that are associated with clinical benefit. Preclinical data have now demonstrated, and clinical data are emerging, on the benefit of combination therapies employing chemotherapy, hormone therapy, small molecule-targeted therapy, and local radiation with vaccine. Several of these additional therapies, when used in appropriate dose-scheduling regimens, have the ability to enhance host immune function and/or alter the phenotype of tumor cells to render them more susceptible to vaccine-mediated T-cell killing.

Autologous Melanoma Cell Vaccine: Establishing Stable Cell Lines With Heterogeneous Tumor-associated Antigens Within a Shorter Duration and Passage

*Senthamil R. Selvan**, Denysha J. Carbonell*, Abner W. Fowler*, Andrea R. Beatty*, Mepur H. Ravindranath†, Robert O. Dillman*. **Cell Biology, Hoag Cancer Center, Newport Beach; †Terasaki Foundation Laboratory, Los Angeles, CA.*

Autologous vaccine, recognized after the failure of melanoma whole-cell and lysate vaccine phase III trials, involves culturing cells from a patient's own tumor within a short duration and with fewer passages but with optimized expression of tumor-associated antigens (TAAs). Its feasibility is established by comparing pure cell lines generated from fresh and cryopreserved tissues (n = 164) of patients with lymph node (LN) and distant metastases. Stable cell lines (from 67% of specimens) were subcultured after cryopreserving them. Pure cell lines established after eliminating fibroblasts (from 96% of the cell lines) include those from LNs (69%), skin (60%), liver (64%), lung (75%), bone (80%), brain 75%, and other sites (73%). Within 3.5 months, stable cell lines (> 50 million cells) were established from initiating the cell culture. For LN metastases, the duration differed significantly ($P^2 < 0.05$) between fresh (1.4 to 3.4 mo) and cry preserved (2.4-4.7 mo) tissues. The expression of TAAs varied: Tyrosinase (81%) > Melan-A (80%) > HMB45/gp-100 (75%) > Mel-5/TRP-1 (65%) > MAGE-1 (47%) > S-100 (28%). The number of TAAs, per cell line, differed between early (< 7) and late (> 7) passages. Among late-passage cell lines, a larger percentage of cell lines expressed 0 to 2 antigens whereas a a lesser percentage of cell lines expressed 3 to 6 antigens suggesting that early-passage (< 7) cell lines are needed for antigen-targeted immunotherapy. This study provides a protocol for establishing cell lines within 2 to 5 months for personalized vaccine therapy.

Effects of Melanoma-derived Helper Peptides and Cyclophosphamide on the Immunogenicity of a Multipeptide Melanoma Vaccine

*Craig Slingluff**, Gina Petroni*, Kimberly Chianese-Bullock*, Mark Smolkin*, Merrick Ross†, Naomi Haas‡, Kelly Smith*, Cheryl Murphy*, Walter Olson*, Nadedja Galeassi*, William Grosh*. **University of Virginia, Charlottesville, VA; †MD Anderson Cancer Center, Houston, TX; ‡Fox Chase Cancer Center, Philadelphia, PA.*

Purpose: In earlier experience, a vaccine mixture of 6 melanoma helper peptides restricted by human leukocyte antigen-DR antigens (6MHP) generated Th1-dominant $CD4^+$ T-cell responses and was associated with durable clinical responses. We hypothesized that combination immunotherapy with 6MHP plus a mixture of 12 class I major histocompatibility complex-associated melanoma peptides (12MP) would augment $CD8^+$ T-cell responses compared with vaccination of 12MP plus a tetanus helper peptide. We also tested the effect of cyclophosphamide (CY) pretreatment on $CD4^+$ and $CD8^+$ T-cell responses.

Experimental Design: A 4-arm randomized phase 2 clinical trial was performed at 3 participating institutions, enrolling 170 patients with resected stage IIB-IV melanoma, who were vaccinated with 12MP plus either a tetanus peptide or 6MHP to stimulate $CD4^+$ T cells, emulsified in an incomplete Freund adjuvant, with or without pretreatment with CY 300 mg/m^2. T-cell responses were assessed by interferon-γ enzyme-linked immunosorbent spot assay in 167 eligible patients (98%), and were defined as increases of at least 20 cells/100,000 $CD4^+$ (or $CD8^+$) cells, at least 2 × negative controls, no overlap of standard deviations with negative controls, and at least 2 × baseline. Weighted mean CVs for normal donor responses to CEF peptide pool were 10% for high responders and 34% for low responders. Clinical outcomes were also recorded.

Results: $CD8^+$ T-cell responses were induced to each of the 12 class I major histocompatibility complex-restricted peptides. The $CD4^+$ T-cell response rates to tetanus peptide and to 6MHP pool were 95% and 53%, respectively. $CD8^+$ T-cell response rates to the 12MP pool (by day 50) were 74% when administered with tetanus peptide (78% and 71% for arms A and B, respectively), but were only 23% when administered with 6MHP (19% and 28% for arms C and D, respectively). CY pretreatment, in arms B and D, had minimal impact on $CD4^+$ or $CD8^+$ T-cell responses. Three-year overall survival estimate (95% confidence interval) was 86% (79%-91%), with longer follow-up needed for enough events to assess outcome by study group.

Conclusions: High immune response rates for these multipeptide vaccines were achieved, but the findings challenge the value of adding melanoma helper peptides to a multipeptide vaccine administered in an incomplete Freund adjuvant. The data also challenge the benefit of CY pretreatment. However, forthcoming data on clinical outcome by arm may modify those conclusions. Future studies will explore helper T-cell subsets and antigen-specific regulatory T cells induced by 6MHP. Nonetheless, overall clinical outcome is promising, across all study arms.

The Synthetic Thiazoloquinolone Immunostimulatory Compound CL075 Serves as a Toll-like Receptor 7/8 Agonist and Substitutes for R848 to Produce Mature Human Monocyte-derived Dendritic Cells With the Capacity to Secrete High Amounts of Interleukin-12(P70) Cytokine

*Stefani Spranger**, Miran Javorovic*, Maja Buerdek*, Susanne Wilde*, Stephanie Tippmer†, Iris Bigalke†, Christiane Geiger*, Bernhard Frankenberger*, Dolores J. Schendel*. **Institute of Molecular Immunology; †GMP Unit, Institute of Molecular Immunology, Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Munich, Germany.*

Cancer immunotherapies based on dendritic cell (DC) vaccination now preferably use mature DC (mDC). mDCs prepared with a

4-cytokine cocktail have been used most frequently in early-phase clinical studies for vaccination of patients with various forms of malignancy. However, these mDCs do not produce bioactive interleukin-12(p70), a critical cytokine for the induction of Th1 cells and activation of natural killer cells. The discovery that toll-like receptor (TLR) signaling in murine DC could activate the nuclear factor-κB pathway opened the door for use of synthetic TLR agonists to modulate the cytokine profile of mDC. We described earlier DCs matured with cocktails that included the TLR7/8 agonist, R848, in combination with the TLR3 agonist poly (I:C) (Zobywalski et al. *J Trans Med*. 2007). The resultant 7-day mDC produced substantial amounts of IL-12(p70) and displayed phenotypic and functional characteristics desired for clinical application, including high cell recoveries. Limited availability of R848 and 7-day culture periods in a GMP facility strongly limited further clinical study development. To overcome these 2 hindrances, we investigated alternatives to R848 and the use of 3-day protocols to rapidly produce mDC. We found that maturation cocktails containing the synthetic thiazoloquinolone immunostimulatory compound CL075 as a TLR7/8 agonist produced mDC within 3 days that showed excellent profiles for clinical application with respect to recovery, phenotype, cytokine secretion, and migration as well as the capacity to activate natural killer cells and effectively polarize $CD4^+$ and $CD8^+$ T cells to secrete interferon-γ. Thus, these 3-day mDCs are highly suited for the development of DC-based antipathogen or antitumor vaccines.

Ad-E2A Preventive and Therapeutic Immunizations Confer Tumor Protection or Delay Tumor Growth in Transplantable HER-2+ Mouse Tumor Models

*Chiara Triulzi**, Simona Vertuani*, Claudia Curcio†, Josef Seibt‡, Federica Cavallo†, Wei-Zen Wei§, Rolf Kiessling*. **Oncology-Pathology, Karolinska Institute, CCK, Stockholm; ‡Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden; †Clinical and Biological Sciences, University of Turin, Turin, Italy; §Karmanos Cancer Institute, Wayne State University, Detroit, MI.*

Her-2 is an oncogenic tumor-associated antigen overexpressed in several human tumors including breast and ovarian cancer. The efficacy and mechanism of a Her-2 – expressing recombinant adenoviral construct (Ad-Her-2) as a preventive or as a therapeutic vaccine was examined in Balb-c wild type, Fc-γ RI/III KO, and B-cell – deficient mice. In all, 108 Ad-Her-2 pfu/mouse were injected once intraperitoneally, and a recombinant adenovirus (Ad) carrying the prostate-specific antigen (PSA) gene was used as a control.

Preventive and therapeutic vaccination of Balb-c mice could, respectively, fully or partially protect the mice from the development of Her-2 – expressing tumors, and correlated with the production of Her-2 – specific antibodies (with a major proportion of IgG2a) and cytotoxic T lymphocytes. Similar results were obtained in hu-Her2 transgenic Balb-c mice, in whom approximately 60% of the vaccinated mice were fully and long-term protected after preventive vaccination.

Results obtained from immunization of Balb-c mice, followed by $CD4^+$ or $CD8^+$ cells in vivo depletion, suggested that the effects were CD8 independent, whereas an antibody-mediated effect was confirmed by therapeutic vaccination of Fc-γ RI/III KO and B-cell – deficient mice. These data, together with the consistent production of IgG2a, strongly suggest that an ADCC mechanism was responsible for the protective effects.

Impact of Low-dose Cytoxan on Circulating Melanoma Antigen-specific T Regulatory Cells In Vivo

*Hideki Ueno**, Laure Bourdery*, Joseph W. Fay*,†, Jacques Banchereau*, A.Karolina Palucka *. **Baylor Institute for Immunology Research; †Sammons Cancer Center, Dallas, TX.*

We have previously reported the detection of melanoma antigen-specific T regulatory cells (Tregs) in the blood of metastatic melanoma patients by EpiMax. NY-ESO-1 – specific Tregs were detected in the blood in 8 of 16 patients with metastatic melanoma. Melanoma antigen-specific $CD4^+$ Tregs secrete interleukin (IL)-10 in response to the epitope stimulation, and express FoxP3. They proliferate in the peripheral blood mononuclear cell culture upon stimulation with their specific epitopes. They suppress the proliferation and the cytokine secretion of $CD4^+$ $CD25^-$ T cells in a cell-to-cell contact-dependent manner. (Vence et al. *PNAS*. 2007;104:20884-20889). EpiMax analysis with NY-ESO-1 overlapping peptides (15 mers) of samples from a new cohort of metastatic melanoma patients (22 patients) revealed that 16 of them (72%) displayed circulating NY-ESO-1 – specific IL-10 – producing $CD4^+$ T cells. Measurement of multiple cytokines revealed that NY-ESO-1 – specific IL-10 – producing $CD4^+$ T cells were heterogeneous, and produced different sets of cytokines. Among the 32 NY-ESO-1 peptides that induced IL-10 secretion in the cultures of the peripheral blood mononuclear cells of 16 patients, 13 induced the secretion of IL-10 alone, 15 induced both IL-10 and interferon-γ, and 4 induced both IL-10 and IL-13. We have used these identified epitopes to assess the impact of low-dose cytoxan administration (300 mg/m^2 intravenous) on the fate of melanoma antigen-specific Tregs. We analyzed the samples obtained at pre-cytoxan and post-cytoxan (day 3) from 10 melanoma patients who displayed NY-ESO-1 – specific Tregs at baseline. A cytoxan injection did not affect the overall frequency of $CD25^+FoxP3+$ Tregs in the blood. Three patients showed decreased IL-10 secretion and the proliferating $FoxP3++$ $CD4^+$ T-cell population after cytoxan in response to the NY-ESO-1 epitopes. On the contrary, 5 patients showed an increased IL-10 secretion and/or the proliferation of $FoxP3++$ $CD4^+$ T cells. Two patients did not show the difference between pre-cytoxan and post-cytoxan. Intriguingly, decrease of IL-10 secretion by cytoxan was limited to specific Tregs secreting IL-10 alone. Such a mixed response to cytoxan might explain the lack of consensus regarding its effects on Treg depletion. The impact on Tregs seems to vary among the patients, which may be relevant to the type of Tregs in each patient. Alternatively, the difference of the outcome may be due to the kinetics of the effect of cytoxan.

Adenovirus-infected Human Dendritic Cells Activate Natural Killer Cells via Plasma Membrane-bound Tumor Necrosis Factor and Interleukin-15

*Lazar Vujanovic**, David E. Szymkowski†, Nikola L. Vujanovic‡,§, Lisa H. Butterfield*,§,‖. **Medicine; ‡Pathology; §Immunology; ‖Surgery, University of Pittsburgh, Pittsburgh, PA; †Xencor, Monrovia, CA.*

Natural killer (NK) cells and dendritic cells (DCs) play important roles in the regulation of innate and adaptive immune mechanisms. Their maturation and activation levels are reciprocally modulated by their cytokine crosstalk. Adenovirus (AdV)-engineered DCs (Ad.DCs) are potent immunologic adjuvants that show promise as a vaccine strategy for the prevention and treatment of viral infections and cancer. The effectiveness of Ad.DC-based vaccines might depend on the ability of Ad.DC to crosstalk with NK cells and to activate, polarize, and bridge innate and adaptive immunity. Here, we investigated for the first time whether and how human Ad.DCs activate NK cells. We performed coculture experiments, using human monocyte-derived Ad.DC and resting NK cells. We found that AdV induced increased expression of transmembrane (tm) tumor necrosis factor (TNF) and trans-presented (trans) interleukin (IL)-15, and increased the ability of DC cells to crosstalk with and activate NK cells, without enhancing DC susceptibility to NK cell killing. The crosstalk yielded enhanced NK cell CD69 expression, interferon-γ secretion, proliferation, and tumoricidal activity. The Ad.DC/NK cell crosstalk was largely prevented by the physical separation of DC and NK cells, and neutralization of TNF and IL-15. In contrast, Ad.DC did not secrete bioactive levels of soluble TNF and soluble IL-15 at the

time of coculture set-up, and selective sequestration of soluble TNF did not prevent Ad.DC/NK cell crosstalk. These findings show for the first time that Ad.DC can efficiently promote innate immune functions, in addition to adaptive immunity, by direct crosstalk with NK cells by cell-to-cell contact, via tmTNF and trans-IL-15.

Active-specific Vaccination Following Immune Depletion With Cyclophosphamide and Subsequent Immune Reconstitution Augments Induction of Tumor-specific T Cells in a Gastric Cancer Mouse Model

*Hauke Winter**, Margareta Rusan*, Dominik Rüttinger*, Nina Schupp*, Wolfgang Zimmermann†, Rudolf A. A. Hatz*, Natasja K. K. van den Engel*. **Department of Surgery, Campus Grosshadern; †Laboratory of Tumor Immunology, Campus Grosshadern, Medical Center, Ludwig Maximilian University of Munich, Munich, Germany.*

Aim: Over 50% of the patients with gastric cancer develop recurrent disease within 5 years after surgery. Active specific vaccination during lymphopenia-driven T-cell proliferation is an innovative strategy with therapeutic efficacy in preclinical models of breast cancer and melanoma. We evaluated this therapeutic strategy in a subcutaneous tumor model for gastric cancer.

Materials and Methods: C57BL/6 mice were treated with or without cyclophosphamide (200 mg/kg, intraperitoneally) to induce lymphopenia 1 day before subcutaneous (SC) vaccination with 10×10^6 irradiated gastric cancer cells (mGC8). Mice received 1 μg mGM-CSF in incomplete Freund adjuvant and were reconstituted with naive C57BL/6 spleen cells (20×10^6 intravenous). After SC injection of 3×10^6 mGC8, tumor growth was measured weekly. To evaluate the tumor-specific immune responses, tumor vaccine-draining lymph nodes (TVDLN) were harvested 8 days after vaccination. TVDLN cells were activated in vitro with anti-CD3 and interleukin-2. Effector TVDLN cells were cocultured with mGC8 cells or syngeneic control tumor. Tumor-specific interferon-γ and interleukin-5 release was determined by enzyme-linked immunosorbent assay. Spleen cells were analyzed for the expression of CD4, CD25, and Foxp3 as markers for regulatory T cells by flow cytometry.

Results: We found an improved protection against SC tumor growth when lymphodepletion and immune cell reconstitution preceded tumor vaccination. The combination of lymphopenia induction and GM-CSF application to the tumor vaccine strongly augmented the induction of tumor-specific T cells as determined by their tumor-specific interferon-γ release. Concurrent with the induction of tumor-specific T cells, we observed an increase of $CD4^+$, $CD25^+$, FoxP3+ T cells, which were able to suppress tumor-specific immune responses.

Conclusions: In contrast to our expectations, the selected therapeutic regimen not only induced tumor-specific T cells but also promoted the recruitment of regulatory cells. Further investigations will focus on the improvement of antitumor immune responses by inhibiting regulatory T cells.

Powerful Induction of Natural Killer Cell Tumoricidal Functions by Type-1 Polarized Dendritic Cell: Positive Feedback Between DC and NK Cells

*Jeffrey L. Wong**, Adam Giermasz†, Robbie B. Mailliard*,†, Ravikumar Muthuswamy†, Eva Wieckowski†, Michael T. Lotze*,†, Pawel Kalinski*,†. **Immunology; †Surgery, University of Pittsburgh and the University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

Dendritic cells (DCs) are the key inducers and regulators of antitumor immunity involved in intercellular communication among different types of immune cells. Previously, we have shown that 2-signal – activated "helper" natural killer (NK) cells induce type-1 polarization of dendritic cells (DCs), enhancing their ability to promote T_H1 and cytotoxic T lymphocyte responses (*J Exp Med.* 202:941–995). Here we show that type-1 – polarized DCs (DC1s) show strongly enhanced effectiveness in promoting antitumor activity of NK cells. Compared with immature or mature but nonpolarized DCs, DC1s induce higher NK cell production of interferon-γ and higher cytotoxicity against tumor targets. The observed effects are largely dependent on the elevated interleukin-12/23 production by polarized DC1s, but involve the participation of additional factors. Combined with evidence that recently activated NK cells are effective in inducing DC1s, these data indicate the existence of a positive feedback loop between polarized DC1s and NK cells involved in immunity against major histocompatibility complex class I-deficient tumor variants and provide a rationale for the combined use of DC and NK cell-targeting strategies in cancer immunotherapy.

Abrogation of Local Cancer Recurrence After Radiofrequency Ablation by Dendritic Cell-based Hyperthermic Tumor Vaccine

He-Xin Yan, Qiong Liu, Hong-Yang Wang. *International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Hospital, Shanghai, China.*

Local recurrence is a therapeutic challenge for radiofrequency ablation (RFA) in the treatment of small solid focal malignancies. Here we show that RFA-induced heat shock protein expression

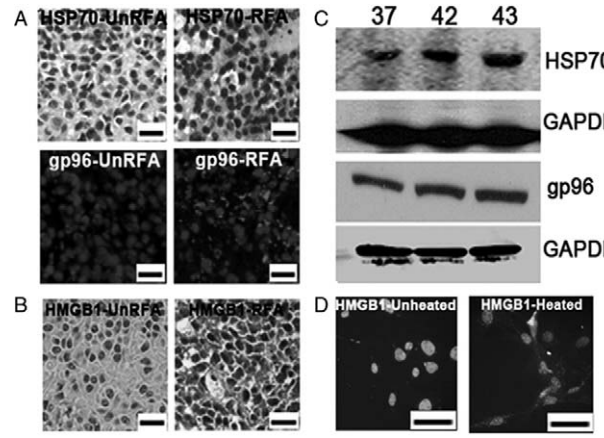

FIGURE 1 (Yan).

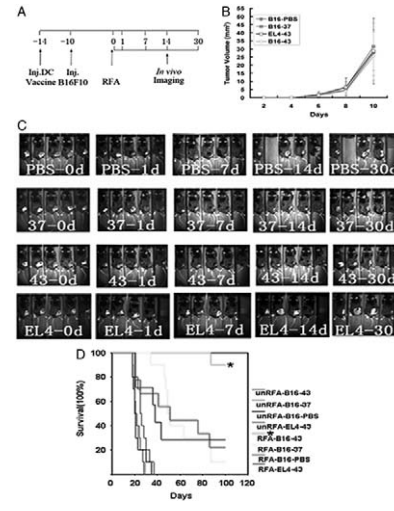

FIGURE 2 (Yan).

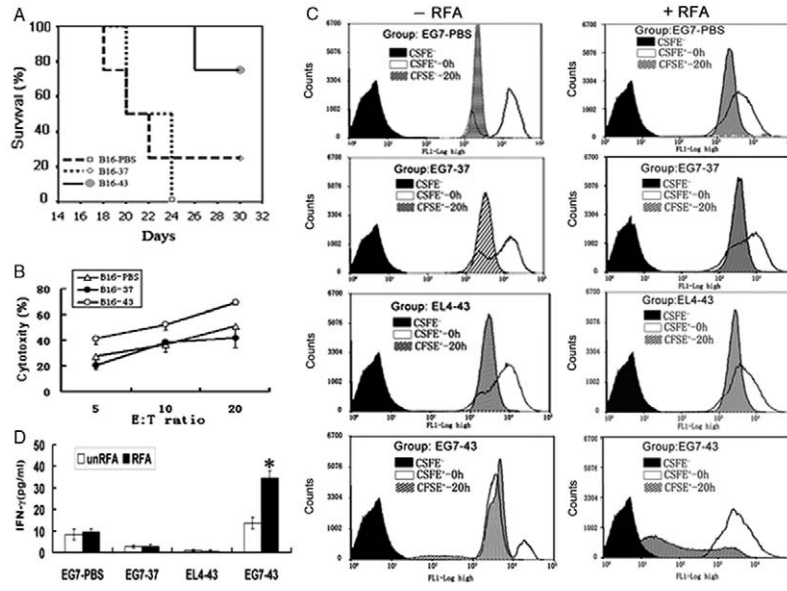

FIGURE 3 (Yan).

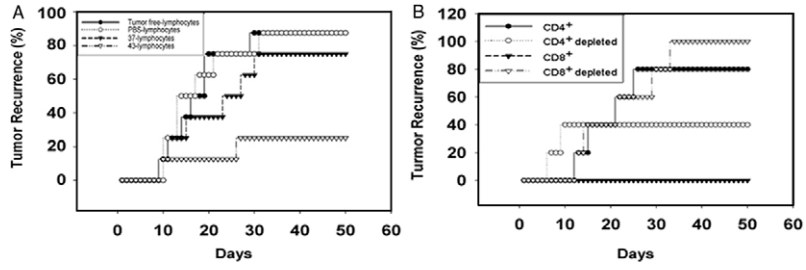

FIGURE 4 (Yan).

and high mobility group box 1 translocation in xenografted melanoma (Fig. 1), which might create a proinflammatory microenvironment that favors tumor antigen presentation and activation of the effector T cells. On this basis, we investigate whether a prime-boost strategy combining a prime with heat-shocked tumor lysate-pulsed DC (HT-DC) followed by an in situ boost with radiofrequency thermal ablation can prevent local tumor recurrence. The combination treatment with HT-DC and RFA showed potent antitumor effects, with $\geq 90\%$ of tumor recurrence abrogated after RFA treatment (Fig. 2). In contrast, prevaccination with unheated tumor lysate-pulsed DC had little effect on tumor relapse. Analysis of the underlying mechanism revealed that splenocytes from mice treated with HT-DC plus RFA contained significantly more tumor-specific, interferon-γ – secreting T cells compared with control groups (Fig. 3). Moreover, adoptive transfer of splenocytes from successfully treated tumor-free mice protected naive animals from tumor recurrence after RFA, and this was mainly mediated by $CD8^+$ T cells (Fig. 4). Therefore, the optimal priming for the DC vaccination before RFA is important for boosting antigen-specific T-cell responses and prevention of cancer recurrence.

Oncolytic Herpes Simplex Virus Activation of FLT3-ligand Generated Dendritic Cells for Cancer Immunity

*Cecile Zaupa**†, Samuel Rabkin*†. **Brain Tumor Research Center, Massachusetts General Hospital;* †*Harvard Medical School, Boston, MA.*

Oncolytic herpes simplex virus (oHSV) vectors selectively replicate in and kill cancer cells while sparing normal tissue, and infection of tumors in vivo induces a robust $CD8^+$ antitumor immune response. Therefore, we hypothesized that oHSV infection induces "immunogenic tumor cell death," which could be exploited to activate dendritic cells (DCs) to mount an antitumor immune response. For these studies we have used the Flt3-ligand to generate FL-DCs from mouse bone marrow progenitors in vitro. FL-DCs consist of a mixed population of conventional (cDC) and plasmacytoid (pDC) cells. Mouse N18 neuroblastoma cells were infected with G47Δ, a third-generation oHSV, and were used to pulse immature FL-DCs. Analysis of cell surface markers by FACS demonstrated that pulsing with G47Δ-infected N18 cells induced cDC and pDC maturation to a much greater extent than mock-infected N18 cells. Only G47Δ-infected N18 cells induced IL12 and IFNα secretion from FL-DCs. FL-DCs pulsed with G47Δ-infected cells were able to protect mice against a lethal subcutaneous N18 tumor cell challenge, whereas mock-pulsed DCs had no effect.

Supernatants from G47Δ-infected N18 cells were just as effective in pulsing FL-DCs as infected cells. Those vaccinated mice surviving tumor challenge were protected against a subsequent challenge 4 months later with an increased dose of N18 tumor cells, including intracerebral challenge, and against Neuro2A cells, a related neuroblastoma cell line, but not against SCK mammary carcinoma cells. Moreover, vaccination induced strong CTL activity against N18 in vitro. As the infected cell supernatants contain a low dose of G47Δ, we tested whether the addition of low dose G47Δ to mock-infected supernatants could induce immunity. Low-dose infection of

FL-DCs induces maturation of cDCs and pDCs. Vaccination with FL-DCs pulsed with mock N18 supernatant + low-dose G47Δ induces some tumor protection, but much less than FL-DCs pulsed with G47Δ-infected N18 supernatants. As this may be due to the lack of sufficient N18 antigens in the mock supernatant, we combined N18 cell lysates with low-dose G47Δ and these pulsed FL-DCs were just as effective as G47Δ-infected N18 supernatant pulsed FL-DCs in protecting mice. Therefore, G47Δ-infected cells or supernatants are an excellent source of tumor antigens to load and activate FL-DCs, which are then able to induce long-term protection against lethal tumor challenge. In addition, G47Δ can be exploited as an adjuvant with tumor cell lysates to efficiently activate FL-DCs.

HUMAN IMMUNOLOGY

Dendritic Cells as Therapeutic Vaccines in Cancer

*Jacques Banchereau**, Hideki Ueno*, Joseph W. Fay*†, A. Karolina Palucka*. **Baylor Institute for Immunology Research; † Baylor Sammons Cancer Center, Dallas, TX.*

Cancer immunotherapy seeks to mobilize a patient's immune system for therapeutic benefit. It can be passive, that is, transfer of immune effector cells (T cells) or proteins (antibodies); or active, that is, vaccination. Early clinical trials testing vaccination with ex vivo-generated dendritic cells (DCs) pulsed with tumor antigens provide a proof-of-principle that therapeutic immunity can be elicited.

In our center, between March 1999 and February 2005, 64 patients with metastatic melanoma were treated with DC vaccines in the course of 4 phase I/IIa clinical trials. DCs were generated either from $CD34^+$ hematopoietic progenitors or from blood monocytes. Forty-nine human leukocyte antigen-A*0201+ patients received vaccines pulsed with melanoma antigen-derived peptides. Twenty-one patients received DCs loaded with killed allogeneic tumors regardless of their human leukocyte antigen type. Patients received up to 8 vaccinations with antigen-loaded DCs over a maximum of 7 months.

DC vaccinations were safe and tolerable. Nine of the 64 patients were alive as of January 2008 from 39 to 105 months. Median survival was 17 (95% confidence interval, 12–26) months. Preliminary analysis demonstrated the induction of long-lived melanoma antigen-specific $CD8^+$ T cells in a patient who obtained durable complete regression of in-transit melanoma. DC vaccination expanded circulating MART-1 – specific $CD8^+$ T cells. These could be detected after the fourth and eighth DC vaccination as well as 2.5 years after the last vaccination with DCs. The T cells had predominantly effector memory phenotype.

Yet, the clinical benefit measured by the regression of established tumors in patients with stage IV cancer has been observed in a fraction of patients only. Two immune parameters seem linked to the clinical outcome of the patients: (1) objective clinical response is associated with the induction of melanoma-specific effector cells, and (2) all patients display melanoma-specific interleukin-10-secreting $CD4^+$ T cells regulatory/suppressor function that may counteract effector cells. Thus, we need to identify the next-generation DC vaccines able to generate large numbers of high-avidity effector $CD8^+$ T cells and to overcome the regulatory T cells and the suppressive environment established by tumors, a major obstacle in metastatic disease. Our preclinical studies actually demonstrate that Langerhans cells, a DC subset that is absent in interleukin-4 – generated monocyte DCs, are superior in their capacity to prime high-affinity melanoma-specific $CD8^+$ T cells able to kill authentic tumor targets.

Thus, the ultimate ex vivo-generated therapeutic DC vaccine will be heterogeneous and composed of several subsets, each of which will target a specific immune effector. These ex vivo strategies should help to identify the parameters for DC targeting in vivo, which represents the next step in the development of DC-based vaccination. We have already developed a series of constructs based on anti-DC antibodies and vaccine antigens including a series of cancer antigens. Prototype vaccines with viral antigens have already been tested in nonhuman primates and demonstrated immunogenicity. Preclinical studies with cancer vaccines are ongoing. We foresee DC vaccination to be based on in vivo targeting of DCs with fusion proteins containing anti-DC antibodies, and antigens from tumor stem/propagating cells and DC activators.

Independent Regulation of the Effector Function and Chemokine Responsiveness of $CD8^+$ T Cells Versus T-cell Expansion by Dendritic Cell-based Vaccines

*Erik Berk**, Payal B. Watchmaker*, Ravikumar Muthuswamy*, Robbie B. Mailliard*, Julie Urban*, Pawel Kalinski*†‡. **Departments of Surgery; † Immunology; ‡Cancer Institute, University of Pittsburgh, Pittsburgh, PA.*

Introduction: The effective induction of tumor-specific $CD8^+$ T cells by dendritic cells (DCs) in the circulation of cancer patients has been shown to be insufficient for the clinical effectiveness of cancer vaccines. As the maturation status of DCs is important for the type of immune response they induce, we tested whether the induction of cytotoxic T lymphocyte (CTL) functions in $CD8^+$ T cells and the induction of their tumor-relevant homing properties are differentially regulated by different sets of DC.

Methods: To delineate the requirements for the effective expansion of $CD8^+$ T cells and their acquisition of effector functions, we compared the outcome of $CD8^+$ T-cell priming by DCs induced to mature by mediators of acute inflammation (combination of interferons and toll-like receptor ligands) or by mediators of chronic inflammation (presence of prostaglandin E_2).

Results: Both "standard" DCs (matured in the presence of prostaglandin E_2) and type-1 – polarized DCs (matured in the presence of interferons and toll-like receptor ligands) are similarly effective in inducing $CD8^+$ T-cell expansion and acquisition of CD45RO + IL-7Rα + IL-15Rα + phenotype. However, granzyme B expression, acquisition of CTL activity, and peripheral tissue-type chemokine responsiveness (expression of CCR5 and responsiveness to CCL5/RANTES and expression of CXCR3) are features exclusively exhibited by the $CD8^+$ T cells activated by type-1 – polarized DCs.

Conclusions: Our data help to explain the dissociation between the ability of cancer vaccines to induce high numbers of tumor-specific $CD8^+$ T cells in the blood of cancer patients and their ability to promote clinical responses. They also provide for improved strategies of cancer immunotherapy, by helping us to induce tumoricidal CTLs with tumor-relevant homing properties.

Increased Level of $CD4^+FOXP3+$ Regulatory T Cells and Decreased Level of Dendritic Cells in Multiple Myeloma Patients

*Marie K. Brimnes**, Anette Vangsted†, Lene M. Knudsen‡, Hans E. Johnsen§, Inge M. Svane*‖. **Center for Cancer Immune Therapy, Department of Hematology; ‖Department of Oncology, Herlev University Hospital, Herlev; † Department of Hematology, Roskilde Hospital, Roskilde; ‡Department of Hematology, Odense University Hospital, Odense; §Department of Hematology, Aalborg Hospital, University of Aarhus, Aalborg, Denmark.*

Patients with multiple myeloma suffer from a general impaired immunity comprising deficiencies in humoral responses, T-cell responses as well as dendritic cell (DC) function. Thus, to achieve control of tumor growth through immune therapy constitutes a challenge. Careful evaluation of the immune status in patients with multiple myeloma seems crucial before active immune therapy.

In this study, we evaluated the level of both DCs and T regulatory cells (Tregs) in patients with multiple myeloma. Blood samples from patients with multiple myeloma at diagnosis and in remission as well as patients with monoclonal gammopathy of undetermined significance (MGUS) were analyzed and compared with age-matched control donors. We found that the proportion of both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in patients at diagnosis were lowered compared with control donors. However, for patients in remission and patients with MGUS, the picture was more complex. The proportion of pDCs in both patients in remission and with MGUS was significantly lower than in controls whereas the proportion of mDCs was not significantly different

from either the patients at diagnosis or in controls. Thus, clearly both types of DCs were influenced in the newly diagnosed patients whereas it seems more unclear how MGUS and lowered tumor burden affect the DC compartment.
Next, we evaluated the level of Tregs in the same patient groups and found that the proportion of $CD4^+FOXP3+$ T cells was increased only in patients at diagnosis. Thus, it seems that Tregs are only increased in patients heavily affected by their disease. In conclusion, our results illustrate that mDCs, pDCs, and Tregs are affected in patients with multiple myeloma underlining the fact that the immune system is dysregulated as a consequence of the disease.

Identification of Colon Cancer-associated Antigens, Which Would be Key Therapeutic Targets in the Prevention of Disease Relapse or Progression

*Elizabeth Broussard**, Vy Lai†, Jennifer Childs†, Doreen Higgins†, Elizabeth O'Donoghue†, Kathleen Tietje†, Hailing Lu†, Mary L. Disis†. **Gastroenterology; †Oncology, University of Washington, Seattle, WA.*

Colon cancer is the second leading cause of cancer death in the United States. Recent studies have shown that colon cancer is an immunogenic tumor and that adaptive immunity may play a role in inhibiting disease relapse. Ideally, a vaccine that could boost cellular immunity in patients with colon cancer would allow prevention of disease recurrence in the majority of patients. However, there have been few defined immunogenic proteins, implicated in driving the malignant phenotype, which have been identified as vaccine candidates. Our group has shown that overexpressed tumor-associated proteins, those that are abundant in the cancer state but expressed at basal levels in normal cells, can be immunogenic. We questioned whether biologically relevant immunogenic proteins could be identified for a vaccine aimed at inhibiting colon cancer relapse or progression. We hypothesized that ideal candidate antigens would be those that had already been validated as prognostic markers in multivariate analysis. To that end, we performed a directed literature review using a variety of search words for example, colon cancer, prognostic, biomarker, etc to identify such proteins in colon cancer. From the over 120 papers identified, we selected a panel of 8 proteins based on (1) incidence of expression, (2) independent predictor of poor prognosis, (3) independent predictor of early disease recurrence, and (4) known biologic function in colon cancer pathogenesis. Two of the 8 selected proteins had already been shown to be human tumor antigens. The panel includes proteins involved in cell adhesion, migration, and division, angiogenesis, inhibition of apoptosis, and the evasion of immunologic defense mechanisms. Using an algorithm program developed by our group based on the widely available web-based prediction methods, we identified peptides derived from each candidate protein that were predicted to be high affinity binders across multiple human leukocyte antigen-DR alleles; characteristics we have shown to be associated with native epitopes of self-tumor antigens. Data will be presented on the immunogenicity of these candidate antigens in patients with colon cancer demonstrating that proteins involved in mediating disease progression can serve as immunologic targets.

Harnessing Invariant Natural Killer T Cells to Abolish the Suppressive Activity of Myeloid-derived Suppressor Cells and Restore Melanoma-specific Immune Response

Vincenzo Cerundolo. Nuffield Department of Clinical Medicine, University of Oxford-Institute of Molecular Medicine, Headington, Oxford, United Kingdom.

We have previously shown that the activation of invariant natural killer T (iNKT) cells results in dendritic cell maturation and assists in the priming of antigen-specific T lymphocytes. We have recently extended these results by assessing the priming of B lymphocytes and characterizing the parameters controlling the activation of iNKT cells and binding affinity of iNKT-cell receptor to CD1d/lipid complexes. More recently, we have described that activation of iNKT cells can abolish the suppressive activity of myeloid-derived suppressor cells during viral infection and cancer growth, restoring antigen-specific immune responses. These results are clinically relevant as indicated by the potential benefits of harnessing iNKT cells in cancer vaccination strategies.

T-cell Recognition and the Coming Golden Age of Human Immunology and Immunotherapy

Mark M. Davis. Stanford University School of Medicine/Howard Hughes Medical Institute, Stanford, CA.

T-cell recognition of specific peptide-major histocompatibility complexes is one of the key events in most successful immune responses and thus a desired outcome in many immunotherapeutic approaches to cancer. In a recent study, we have investigated the basic biochemistry of αβ T-cell receptor (TCR)-mediated recognition in solution and in its cellular context, the immunologic synapse. We find a number of remarkable aspects from the organization of TCRs on specific "protein islands" to the molecular dynamics of their interactions within a synapse. These features are likely to be important in the sensitivity and ligand-discriminating qualities of TCRs. In another study, we have investigated the T-cell repertoire in normal humans and find that naive T cells to foreign antigens are present in very similar frequencies to those found in the mouse, despite having $\sim$100 times more T cells, suggesting that the ligand repertoire is regulated in some way. More remarkably, we find that self-antigen reactive T cells are very abundant in peripheral blood and thus a major function of the immune system must be to keep these from causing autoimmunity. We have also begun to develop metrics of what constitutes a normal human immune response, using influenza vaccination as a model. These later efforts are based on the idea that we need to develop a much more extensive knowledge of human immunology, independent of animal models, to correctly navigate the challenges of developing immunotherapies.

The Biology and Therapy of Brain Metastasis

Isaiah J. Fidler. MD Anderson Cancer Center, Houston, TX.

The major cause of death from cancer is metastasis. In the United States, more than 40% of cancer patients develop brain metastasis. The median survival for untreated patients is 1 to 2 months, which may be extended to 6 months with conventional radiotherapy and chemotherapy. The growth and survival of metastases depends on the interaction of tumor cells with host factors in the organ microenvironment.
The brain microenvironment regulates the resistance of tumor cells to systemic therapy. The mechanism for this resistance has been under active investigation. One potential mechanism is the blood-brain barrier (BBB), which protects the normal brain parenchyma from circulating toxic substances. In brain metastases, however, the BBB is permeable. This permeability is due to increased release of vascular endothelial growth factor-vascular permeability factor by tumor cells growing in the brain, suggesting that the resistance of brain metastases to chemotherapy is mediated by a mechanism independent of the BBB.
Histologic examination of clinical specimens of human breast, lung, melanoma, and colon brain metastases shows that the lesions are surrounded and infiltrated by activated astrocytes expressing glial fibrillary acidic protein (GFAP). GFAP-positive astrocytes are also associated with experimental brain metastases produced by lung, brain, melanoma, and colon cancers. We isolated astrocytes from the brain of the "ImmortoMouse" and established the cells in culture.
Astrocytes cocultured with melanoma, breast cancer, or lung cancer cells protect the tumor cells from all tested chemotherapeutic agents (Taxol, VCR, VGL, 5-FU, Cisplatinum, ADR). Establishment of a gap junction between the astrocytes and tumor cells is required for this chemoprotection. Coculture of tumor cells with other tumor cells or fibroblasts does not protect the cells from chemotherapeutic drugs. Microarray experiments for cross-species hybridization (human tumor cells cocultured with mouse astrocytes or mouse fibroblasts) identified up-regulation of several survival genes, including GSTA5,

BCL2-L1, TWIST1, and BCL-xL, in tumor cells cocultured with astrocytes, but not with fibroblasts. Once removed from astrocytes, the expression of these genes declined, and the tumor cells lost the resistance to the chemotherapeutic drugs.
These data clearly show that host cells in the microenvironment influence the biologic behavior of tumor cells and reinforce the contention that the organ microenvironment must also be taken into consideration during the design of therapy.

Multiple Peptide Vaccines in Cancer Patients With Early Disease or Limited Tumor Burden

*Paola Filipazzi**, Agata Cova*, Paola Squarcina*, Felicetta Giardino*, Gianluigi Rigamonti*, Marco Asioli*, Paola Frati*, Giorgio Parmiani†, Licia Rivoltini*. **Fondazione IRCCS Istituto Nazionale Tumori; †IRCCS San Raffaele Scientific Institute, Milan, Italy.*

The optimal use of antitumor vaccines should be in the presence of minimal tumor burden and in early disease, when the immune functions of patients are mostly conserved. To address this hypothesis, we tested a multipeptide vaccine in patients with early melanoma and biochemical recurrence for prostate carcinoma. Stage IIB-C/III melanoma patients were enrolled in a phase II randomized trial (80 patients/arm) of observation versus vaccination with HLA-A*0201-restricted modified tumor peptides [MelanA/MART-1(26-35/27L), gp100(209-217/210M), NY-ESO-1(157-165/165V), Survivin(96-104/97M)] emulsified in Montanide ISA-51. In prostate carcinoma, the phase II trial consisted of vaccination again with HLA-A*0201-restricted peptides, and was planned to include 30 patients with biochemical failure after surgery or radiotherapy (mean prevaccination PSA: 1.83 ng/mL; 0.4–5.07 ng/mL). Treatment consisted of two natural peptides from PSMA (PSMA4-12 and PSMA711-719) and the abovementioned modified peptide, survivin (96-104/97M), emulsified in Montanide ISA 51. In both studies, peptide administration was preceded by low-dose cyclophosphamide (300 mg/m^2), aimed at reducing the frequency and activity of regulatory T cells. As of now, 43 melanoma patients (22 vaccine vs. 21 observation arm) have been enrolled in the melanoma trial, and 20 patients in the prostate carcinoma study. Although patient-to-patient variability was observed in the frequency and magnitude of the response, 75% of tested patients from the vaccinated arm responded to at least one of the epitopes, in both melanoma and prostate carcinoma trials, as detected by IFNα Elispot and HLA-multimer staining. However, the raised recognition of modified peptides observed in postvaccine PBMC was not paralleled by an improved capability of CD8$^+$ T cells to recognize antigen-expressing tumor cells. These results can be explain by the evidence that modified peptides drive the induction of T cells poorly cross-reacting with the native epitopes, thus leading to a low frequency of tumor-reactive T cells in postvaccine PBMC. From a clinical viewpoint, vaccination did not significantly impact disease-free survival in melanoma patients, whereas $>50\%$ of prostate carcinoma patients experienced a significant but transient decrease of PSA serum levels. In conclusion, immunization with modified peptides, while mediating effective boosting of antigen-specific immunity in patients with early disease or low tumor burden, does not produce a sufficiently potent increase of anti-tumor CD8$^+$ T cells to reach persistent clinical efficacy.

γδ T Cells: Agents of Tissue and Tumor Immune Surveillance

Adrian Hayday. King's College School of Medicine at Guy's Hospital, London, and the London Research Institute, Cancer Research UK, London, United Kingdom.

The community has adopted a powerful and compelling description of immune surveillance of infected tissues, which links the activation of myeloid lineage cells [dendritic cells (DC); monocytes, and macrophages] of the innate response to the activation of antigen-specific lymphocytes of the adaptive response. The key initiators are inherited pattern recognition receptors, such as Toll-like receptors (TLRs), and TLR agonists are therefore being applied to enhance tumor immunotherapy. However, nonmicrobial stress, including cell transformation, can induce or otherwise alter the expression of a variety of cell surface molecules, collectively expressed by a broad spectrum of cells, but particularly epithelial cells. The receptors for these "stress-antigens," such as MICA, are frequently found not on myeloid cells, but on "unconventional" or "innate-like" lymphocytes, of which γδ cells are the prototype. Using transgenic mouse systems, we have shown that the induced expression of these stress antigens is sufficient to initiate a "lymphoid stress-surveillance response" that is much more rapid than conventional T-cell responses, and that composes a new component of immunobiology that can limit carcinogenesis. This presentation will describe the key features of lymphoid stress surveillance, including evidence of its capacity to regulate adaptive antigen-specific responses. In this context, we will also review our and other investigators' experience of the clinical application of γδ T cells against prostate cancer and other malignancies. Promising indications suggest that this may be a useful clinical strategy, possibly in combination with cancer vaccines.

Damage and Pathogen-associated Molecular Pattern Molecules Decrease Cytoplasmic Zinc in Human Dendritic Cells and Promote High Mobility Group B1 Cytosolic Translocation

*Erica John**, William Buchser*, Bruce Pitt†, Michael Lotze*. **Surgical Oncology; †Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA.*

Zinc is the second most abundant metal in mammals, and regulates immune function in the T cells and DCs of the immune system.[1] Zinc deficiency and excess are linked with immune dysfunction. The Pathogen-Associated Molecular Pattern (PAMP) LPS decreases DC zinc levels through Toll-like receptor signaling.[2] The dependence of human peripheral blood monocyte-derived DCs on zinc homeostasis was explored in 2 ways. First, we manipulated zinc in DCs with chelators, ionophores, and the addition of zinc to the media at various concentrations. We also measured intracellular zinc after exposing cells to damage-associated molecular patterns (DAMPs) and PAMPs with fluozin. Preliminarily, exposure of DCs to DAMPs or PAMPs lowers intracellular zinc, comparable to the chelator TPEN. The addition of LPS and manipulation of zinc increases the cytoplasmic localization of HMGB1. DC maturation and ability to induce an immune response is dependent on HMGB1 translocation and reduced high-mobility group B1 (HMGB1) in the extracellular environment. Targeting and measuring Zn and HMGB1, as well as Zn receptors and RAGE during immunotherapy regimens are likely important biomarkers and means to enhance therapy.[2]

References:

1. Kitamura H, Morikawa H, Kamon H, et al. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. *Nat Immunol*. 2006;7:971–977.
2. Sparvero LJ, Asafu-Adjei D, Kang R, et al. RAGE (Receptor for Advanced GlycationEndproducts), RAGE ligands, and their role in cancer and inflammation. *J Transl Med*. 2009;17:7–17.

Immune Regulation in Endometriosis and Endometriosis-related Ovarian Cancer

Gina Mantia-Smaldone†, Raluca Budiu*†, Ted Lee†, Suketu Mansuria†, Kristin Zorn*†, Robert P. Edwards*†, Anda Vlad*†. **Obstetrics, Gynecology and Reproductive Sciences, Magee-Women's Research Institute Pittsburgh, PA; †Obstetrics, Gynecologic and Reproductive Sciences, Magee-Women's Hospital/University of Pittsburgh Medical Center, Pittsburgh, PA.*

Endometriosis is a chronic, benign disease relying on aberrant survival of ectopic tissue resembling endometrial glands and stroma. Endometriosis is associated with an increased risk for endometrioid and clear-cell epithelial ovarian cancers (EOC). Prevention strategies for ovarian cancer, the most lethal gynecologic malignancy, are a research priority, and early identification and treatment of precursor lesions is essential for long-term

survival. Although the exact causes remain largely unknown, it is now believed that endometriosis is partly mediated by impaired immune mechanisms in the host. Although innate immunity has been explored earlier, antigen-specific adaptive immune responses have not been identified due to a limited number of identifiable endometriosis antigens. We postulated here that MUC1 glycoprotein, a known ovarian tumor-associated antigen, is also present in ectopic ovarian endometriosis lesions and that changes in MUC1 expression affect its immunogenicity, similar to those seen in ovarian cancer. Furthermore, we hypothesize that endometriosis is associated with an imbalance between CD4 T-cell subsets, which may contribute toward endometriosis pathogenesis and ovarian carcinogenesis. To test this hypothesis, we obtained biologic specimens from 27 women with endometriosis, 10 with endometriosis-related (endometrioid and clear cell) EOC, and 15 healthy controls, according to University of Pittsburgh institutional review board-approved protocols. We measured MUC1 expression via immunohistochemistry and anti-MUC1 IgM and IgG antibody levels by enzyme-linked immunosorbent assay. Peripheral blood $CD4^+$ T-cell subsets were immunophenotyped by multicolor flow cytometry. Our preliminary results identify MUC1 expression in endometriosis and identify tissue infiltration of CD4, CD4 FOXP3, and CD8 T cells. Furthermore, women with endometriosis and endometriosis-related EOC have similar anti-MUC1 IgM and IgG antibody responses. Th1, Th17 and T regulatory responses are present in women with endometriosis, and women with endometriosis seem to have higher T regulatory and Th17 and similar Th1 peripheral blood T-cell populations in comparison with women with endometriosis-related EOC. Our ongoing analyses of antigen-specific immunity in the lesion microenvironment will increase our understanding of immune pathogenesis of endometriosis and may contribute to immune biomarker discovery and improvements in the early detection and prevention of ovarian cancer.

Relationship Between Psychological Stress and Immunosuppressive Cell Populations in Breast Cancer Patients

*Bethany L. Mundy**, Lisa M. Thorton†, Barbara L. Andersen†, William E. Carson‡. **Integrated Biomedical Sciences; †Psychology; ‡Surgery, Ohio State University, Columbus, OH.*

Our group has recently shown in a randomized clinical trial that psychologic intervention to reduce stress in patients with stage 2 and 3 breast cancer led to enhanced immune functioning, fewer recurrences, and improved overall survival. Patients in the Intervention arm were found to have a reduced risk of breast cancer recurrence (HR of 0.55; $P = 034$) and death from breast cancer (HR of 0.44; $P = 016$) compared with patients in the Assessment-only arm. We hypothesized that elevated levels of cytokines associated with chronic stress could lead to an immunosuppressive environment in these patients. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of early myeloid cells that are known to accumulate in patients with cancer and actively inhibit the host antitumor immune response. We hypothesized that patients with high levels of perceived stress at baseline would have alterations in the MDSC compartment compared with patients with lower perceived stress. We evaluated 16 samples from patients. Eight patients had high stress and 8 had low stress after surgery as evaluated by the Impact of Events Scale (IES) questionnaire. Flow cytometric analysis for $CD33^+$/HLA-DR−/$CD15^+$/$CD11b^+$ MDSC revealed increased MDSC in patients with lower IES scores ($P = 0.01$). $CD11b^+$/$CD15^+$ cells constituted 9.4% of the $CD33^+$/HLA-DR− cell population in patients with high IES, whereas this population constituted 27.3% of $CD33^+$/HLA-DR− cells in patients with low IES scores. In addition, patients with low IES scores had elevated plasma levels of interleukin-6 (3.06 pg/mL in the high IES group vs. 2.01 pg/mL in the low IES group) and decreased plasma levels of interleukin-1Rα (45.09 pg/mL in the low IES group vs. 97.16 pg/mL in the high IES group). Patients with high IES scores had elevated salivary cortisol levels (13 μg/dL vs. 9.74 μg/dL), which inversely correlated with MDSC percentages. There was no significant difference in natural killer (NK) cell counts between the 2 groups or ability to lyse the NK-sensitive cell line, K562. The average NK cytotoxicity at the 100:1 target:effector ratio was 50.15 lytic units in low-stress patients versus 48.04 lytic units in high-stress patients. T-cell proliferation in response to PHA and ConA were comparable between high IES and low IES patients (absorbance of 0.05 for PHA and 0.3 for ConA in both groups). These data indicate that a complex relationship exists between stress and immune function in cancer patients. Assessment of additional patients is needed to determine the mechanisms and long-term immunologic consequences of elevations in suppressive cell populations.

Autologous Vaccine AHICE©: An Immunotherapy Patent Against Pancreatic Cancer

*Konstantinos Papapolychroniadis**, Hannes Anthopoulos†, Ioulia Papapolychroniadou‡, Mairi Georgiou*, Daniil Paramythiotis*, Parisis Makrantonakis§, Epaminontas Fahantidis*. **1st Surgical Propedeutic Clinic; §Department of Medical Oncology, First Medical Clinic Aristotle University of Thessaloniki, Thessaloniki, Greece; †K-Bio Clinical Biochemical Institute for Cell Biotechnology and Immunology, Munich, Germany; ‡Department of Health and Human Sciences, London Metropolitan University–Pharmaceutical Science, London, United Kingdom.*

A significant active autologous vaccine, AHICE©, as an antitumor therapy for immunologic pancreatic cancer by a specific biochemical patent (K-BIO) and the elimination of cancer cell escape mechanisms are reported in this study. The immune system then begins to kill the identified target cells (primary and metastatic) apoptotically by a very complex and specific immunologic and biochemical mechanism, which will be analyzed during the presentation. In this study, we present the principles of management and outcomes of a series of 8 pancreatic ductal cancer patients. The period of each long-term immune therapy cycle was 3 months. In 2 patients after the first successful cycle, a second one was followed. No side effects, toxic or other complications were recorded. In a case of advanced disease with intraperitoneal dissemination and ascites, immune therapy was discontinued. We can conclude that AHICE© therapy offers (1) an in vivo effective therapy with no side effects or toxic results; (2) a well-acceptable and high living-quality therapy without complications; (3) an individual/personalized and remarkably optional therapy; (4) Further cases were needed to study in randomized trials.

A Rare Advanced Stage Case of Medullary Thyroid Cancer: Significant Outcome by Autologous Human Immune Vaccine-AHICE Therapy

Konstantinos Papapolychroniadis*, Hannes Anthopoulos†, Epaminontas Fahantidis*, Vasilis Papadopoulos*, *Ioulia Papapolychroniadou‡. *1st Surgical Propedeutic Clinic, Aristotle University of Thessaloniki, Thessaloniki, Greece; †K-Bio Clinical Biochemical Institute for Cell Biotechnology and Immunology, Munich, Germany; ‡Department of Health and Human Sciences—Pharmaceutical Science, London Metropolitan University, London, UK.*

Autologous human vaccine AHICE is a specific antitumor active immune therapy. The main target of this active immune therapy is to make possible the recognition, identification, and killing of masked altered-shape cancer cells, because of their escape mechanisms. This specific apoptotic cycle of tumor cells is succeeded by a specific activation of natural killer cells, which are controlled by the complex sum of the immune response products (eg, cytokine chain, colony-stimulating factors, chemokines, and related effectors).

We present a rare and interesting case of a 38-year-old diagnosed with medullary thyroid cancer on April 2003. After the correct research study, total thyroidectomy plus neck and mediastinal nodal dissection were performed. During the follow-up, several recurrences were discovered and additional nodal and tumor

resectional procedures were performed in 2005, 2006, and January 2007. A right axillary plus ipsilateral supraclavicular nodal dissection was performed in October 2007. On account of the repeated recurrence status, AHICE immune therapy was started in January 2008, in a series of 2 consecutive cycles with successful results, symptom remission, performance status improvement, and significant decrease of calcitonin plasma concentration levels. After the completion of AHICE immune therapy, there was an indication of radical dissection, which was performed in March 2009. Unfortunately, the patient succumbed suddenly from cardiorespiratory arrest 1 month later.

In conclusion, we address the effectiveness of AHICE immune therapy in rare, interesting, difficult, and multiregional recurrence cases of medullary thyroid cancer patients.

A Data Analysis Method for Identifying Autoantibody Biomarkers in Cancer Patients Following Immunotherapy

*Janet Siebert**, Sachin Puri†, James A. Thompson†, Tarsem Moudgil†, Ilka Assmann†, Theresa Ratzow†, Shawn Jensen†, Michael Lacelle†, Daniel Haley†, Christian A. Poehlein†, Edwin Walker†, Nathalie Sacks‡, Kristen Hege‡, Hong-Ming Hu†§‖, Brendan Curti†, Walter J. Urba†, Bernard A. Fox†‖¶. **CytoAnalytics, Denver, CO; ‡Cell Genesys Inc, South San Francisco, CA; †Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center; §Department of Radiation Oncology; ‖OHSU Cancer Institute; ¶Departments of Molecular Microbiology and Immunology; and Environmental and Biomolecular Systems, OHSU, Portland, OR.*

Immunologic monitoring following the administration of allogeneic whole tumor cell vaccines is a complex challenge. Frequently, analysis is limited because autologous tumor cells are unavailable. This is particularly problematic in patients with breast and prostate cancer. One approach to overcoming this limitation is to use protein array technology to monitor the immune response generated after vaccination. However, a major limitation to performing these studies is the absence of a software package for identifying candidate antibody markers. Here we describe a method for data analysis of low-frequency and diverse autoantibody profiles in complex protein array data. Serum antibody reactivity, hereinafter referred to as protein expression, with 8217 autoantigens was measured pretreatment and posttreatment for 11 prostate cancer patients using Invitrogen's ProtoArray Human Protein Microarray. Patients showed diverse protein expression profiles, with the number of "hits" per patient ranging from 37 to 7585 proteins (average = 2133). On account of this diversity of expression, we applied specialized informatic techniques to identify proteins that showed increased or decreased expression after treatment in multiple patients. First, we filtered the data on a per-treatment per-protein basis to limit the analysis to only those proteins with a credible reading in both pretreatment and posttreatment samples. Second, for each donor, we identified the proteins with the 50 largest posttreatment increases and the proteins with the 50 largest posttreatment decreases. Third, we consolidated these "Top 50" lists to determine which proteins increased or decreased in multiple patients. There were a total of 418 distinct proteins in the Top 50 posttreatment increase list, with 46 of those expressed by 2 or more donors, and 13 expressed by 3 or more donors. There were a total of 393 distinct proteins in the Top 50 posttreatment decrease list, with 44 of those expressed by 2 or more donors, and 14 expressed by 3 or more donors. Four of the 11 patients showed increased expression of 3 different galectin clones, making galectin a candidate for further research.

A Novel Software Application for Enzyme-linked Immunosorbent Spot Data Calculation, Conversion, and Quality Assurance Reporting

Meredith Slota, Elizabeth O'Donoghue, Kathleen Tietje, Mary L. Disis. *Center for Translational Medicine in Women's Health, Tumor Vaccine Group, University of Washington, Seattle, WA.*

The enzyme-linked immunosorbent spot (ELISPOT) assay is widely used for immune monitoring in clinical trials of cancer immunotherapy. The flexibility and sensitivity of the assay with a validated protocol makes the method appropriate for high-throughput screening. Generation of bulk ELISPOT data [spots/well (SPW); corrected spots/well (CSPW)] can be cumbersome to manage, as multiple analyses are performed for each patient. In addition, it is important to maintain raw data, such as "no antigen" wells, to assess assay performance for the ongoing quality assurance (QA). Moreover, ELISPOT data are commonly analyzed manually or with spreadsheet methods prone to user error. Our group has created a software tool to collect, analyze, and store ELISPOT data, making high-throughput assays more manageable and allowing for centralized data analysis using uniform calculations. This tool uses a standard 96-well customizable format that enables researchers to export raw data directly into the program. The data conversion function transforms raw SPW data into reportable values such as CSPW or precursor frequency via 3 complex calculation queries that result in both reportable data and figures that can be used for longer-term QA of assay performance. The historical "snapshot" function uses an appended query to store the current calculated values with a date/time or reagent lot stamp and a comment into the historical data snapshot table. When performing analyses, researchers can choose to use the most current dataset or any previous snapshot dataset. This snapshot function allows for a continually evolving database while maintaining archival datasets, allowing for more facile trending of assay performance or biologic variation. The data conversion tool is highly customizable, allowing for changes in common assay variables. By preserving all raw data, we can generate reports on background levels and ensure that positive controls are working consistently. We can also be alerted to outlier assays that may indicate issues in assay performance or clinical trial conduct. The software creates QA/QC reports on changing reagents and monitors performance across different technicians and clinical trials. The creation of this novel software tool has improved our data quality and consistency, analysis efficiency and accuracy, and has assisted in the development of a solid QA program for immunologic monitoring of human clinical trials.

Vaccination of Leukemia Patients With a Major Histocompatibility Complex Class I Peptide of Wilms Tumor Gene 1 (WT1) Peptide With Unspecific T Helper Stimulation is Able to Induce WT1-specific IgM Responses but Fails to Induce IgG Responses

*David Stather**, Carmen Scheibenbogen†, Olga A. Elisseeva‡, Sandra Bauer†, Yoshihiro Oka‡, Haruo Sugiyama‡, Eckhard Thiel*, Ulrich Keilholz*, Anne Letsch*. **Hematology/Oncologie Campus Benjamin Franklin; †Institute of Medical Immunology, Campus Mitte, Charité, Berlin, Germany; ‡Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University, Osaka, Japan.*

Objectives: To assess the vaccine-induced humoral response against a human leukocyte antigen (HLA)-A201-restricted Wilms tumor 1 (WT1) peptide in comparison with an entire protein.

Methods: Analyses were performed in 14 HLA-A2-positive AML patients receiving biweekly vaccinations with 62.5 μg granulocyte macrophage-colony stimulating factor (day 1-4) and on day 3, 0.2 mg of the HLA-A201-restricted WT1.126-134 epitope peptide admixed with 1 mg KLH, injected intradermally and subcutaneously. Dot-Blot assays to detect WT1-specific antibodies were performed as described previously. For the detection of KLH-specific antibodies by enzyme-linked immunosorbent assay, 96-well plates were coated with KLH (50 μg), blocked with BSA, and then incubated with sera (diluted 1:10). Peroxidase-conjugated anti-IgM or anti-IgG antibodies were then detected with ABTS. WT1-specific T cells, identified by intracellular cytokine accumulation, were detected by flow cytometry.

Results: Humoral responses against WT1 were analyzed at baseline and during the course of vaccination. Enhanced anti-WT1-IgM antibodies were detected in 2 of 7 patients in week 18 and in all of the 6 patients in weeks 26 or 30 during vaccination, but never earlier.
In contrast to the IgM responses, no WT1-specific IgG antibody responses could be induced or enhanced by vaccination. In accordance with these data, no WT1-specific $CD4^+$ T-cell response was observed. General $CD4^+$ failure was excluded by determining the KLH-specific humoral response. After vaccination enhanced KLH IgM antibodies were detected in 3 out of 4 patients in week 10 and in all 4 patients in week 18. In week 10, 4 of 8 patients showed an induction of KLH-specific IgG antibodies, and all 4 patients in week 18 or week 26.
Conclusions: Our findings show that probably due to the missing specific T helper cell induction, long-term vaccination with an HLA-A2/major histocompatibility complex class I restricted peptide and granulocyte macrophage-colony-stimulating factor/KLH as adjuvants is only able to induce WT1-specific IgM antibodies, but not IgG antibodies.

Tumor-derived Microvesicles Deliver Immunosuppressive Transforming Growth Factor-β1 to Distinct Immune Cells in Patients With Head and Neck Cancer

*Miroslaw J. Szczepanski**†‡, Malgorzata Czystowska*, Marta Szajnik*, Michael Boyiadzis*, Malgorzata Harasymczuk†, Witold Szyfter†, Theresa L. Whiteside*. **University of Pittsburgh Cancer Institute, Pittsburgh, PA; Departments of †Otolaryngology; ‡Clinical Immunology, Poznan University of Medical Sciences, Poznan, Poland.*

Background: Head and neck squamous cell carcinomas (HNSCC) escape from the host control by inducing immunosuppression, which can be mediated by various mechanisms. The release by tumors of inhibitory cytokines, such as transforming growth factor (TGF)-β1, and immunoinhibitory microvesicles (TMVs) can contribute to tumor progression. The objective of these studies was to investigate the effects of TMV-associated TGF-β1 on immune cell activity.
Methods: We first evaluated by Luminex, the level of TGF-β1 in sera of laryngeal cancer patients (n = 19), normal controls (NCs) (n = 21), and tumor cell supernatants (n = 6). TMVs were isolated by exclusion chromatography and ultracentrifugation from supernatants of HNSCC cell lines (PCI-13 and PCI-30), patients' and NC sera. The expression of TGF-β1 was also evaluated by immunohistochemistry, immunofluorescence and/or western blots in tumor tissues, tumor cell lines, and in TMV isolated from tumor cell supernatants and sera of cancer patients and NC. A flow cytometry method was developed for the isolation of TGF-β1(+) TMV from HNSCC patients' sera using beads coated with B7H3 captured antibodies (Abs). Phycoerythrin-labeled TGF-β1 – specific Abs were used for TMV detection. Chromium (^{51}Cr) release assays were performed using the K562 cells as targets and natural killer (NK)-92 cells as effectors before and after coincubation of NK-92 cells with TMV (20 μg/μL).
Results: Intracellular TGF-β1 expression was observed in both PCI cell lines and in tumor cells in 17 of 19 tissues, but TGF-β1 was not detectable in supernatants of PCI-13 and PCI-30. However, TMV isolated from tumor supernatants and sera of HNSCC patients were positive for TGF-β1 in western blots and by flow cytometry. These TMVs (20 μg/μL) had biologic activity as they inhibited NK cytotoxicity ($P < 0.05$) and decreased NKG2D as well as interferon-γ expression ($P < 0.05$) in NK-92 cells. These effects were partially abrogated ($P < 0.05$) by TGF-β1 – specific neutralizing Ab.
Conclusions: Most HNSCCs express intracellular TGF-β1. TMVs released from HNSCC are also positive for TGF-β1, which is present and detectable on the TMV surface by flow cytometry. This membrane-associated TGF-β1 can be delivered by TMV to distant immune cells, suppress their antitumor function, and thus facilitate tumor escape from the host immune system.

Application of Real-time mRNA Cytokine Assay for Immune Monitoring of Cell-mediated Immune Responses in Breast Cancer

Masato Mitsuhashi*, Pramod Sutrave†, *Charles Wiseman†*. **Hitachi Chemical Research Center, Inc, Irvine; †Wiseman Cancer Research Foundation, Los Angeles, CA.*

We are evaluating a sensitive and high-throughput microassay using whole blood for detecting cell-mediated immune responses in cancer patients. Triplicate 50 μL aliquots of heparinized whole blood are incubated for 4 to 24 hours with various mitogens or antigens as described (Mitsuhashi et al, *Clin Chem*. 2006). After Leukosorb separation, a reverse transcription-polymerase chain reaction assay was performed to quantify changes in cellular mRNA by the identification of cycle thresholds of test versus saline control, normalized against the reference â-actin gene (ACTB). Although some antigens elicit as much as 300-fold increases in mRNA expression, the method allows for highly sensitive and statistically significant characterization of as little as 1.5-fold to 2.0-fold increases in target mRNA species.
We have explored responses in 8 cancer patients and 2 normal persons after obtaining informed consent under a protocol reviewed by the Western institutional review board. Whole-blood aliquots were incubated with selections from a panel, which included heat-aggregated globulin, anti – T-cell receptor antibody, lipopolysaccharide, phytohemagglutinin, zymozan, purified protein derivative, diphtheria/pertussis/tetanus vaccine (DPT), candida, trichophyton, and Pneumovax, as well as freeze-thaw extracts of breast cancer cell line SV-BR-1 (Wiseman et al. *The Online Breast Journal*. In press). We measured mRNA expression for the following cytokines: interferon-γ, IL2, IL 6, IL 8, TNF-α, TNF-SF15, CCL2, CCL4, CCL20, GMCSF, and VEGF among others. Overnight incubation with breast cancer cell line extract produced as much as 10- to 20-fold upregulation of VEGF and CCL20 in both normal control and breast cancer patients, but other cytokine mRNAs were not significantly changed. In general, the breast cancer patients seemed to show a trend for less intense responses than normal controls. In both cancer patients and normal controls, DPT produced statistically significant upregulation of mRNA expression of IL-2, IL-8, and CCL20 and caused > 50% down-regulation of mRNA expression for ACTB and CCL2 in all of the 5 patients. The observation of down-regulation of ACTB response to DPT was unexpected. Pneumovax, in contrast, consistently up-regulated the expression of all cytokine signals except IL-2.
These exploratory studies and others to follow will evaluate the possible use of this test to correlate immune responses with clinically relevant characteristics.

Identification of a Novel $CD8^+CD57^+$ T-cell Subset in Human Melanoma Exhibiting an Incompletely Differentiated Cytotoxic T Lymphocyte Phenotype

Richard Wu, Shujuan L. Liu, Yufeng Li, Patrick Hwu, Gregory Lizee, Laszlo Radvanyi. *Melanoma Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX.*

A number of studies have shown a lack of full cytotoxic T lymphocyte (CTL) differentiation in solid tumors, but the stages of differentiation that are affected are unclear. Mature CTLs have been associated with 2 markers, CD57 and CD56. However, the association between their expressions on $CD8^+$ tumor-infiltrating lymphocytes (TILs) and which of these markers is associated with mature antitumor CTL is unknown. In this study, we used multicolor flow cytometry to track $CD8^+$ molecular markers associated with specific stages of CTL differentiation in TILs from over 30 surgically resected melanoma metastases. Our aim was to determine the differentiation status of $CD8^+$ TILs and the relationship between $CD57^+$ and $CD56^+$ in the $CD8^+$ subset. First, we observed a striking lack of fully mature CTLs with the majority of $CD8^+$ T cells having an early effector-memory phenotype ($CD27^+$, $CD28^+$, $CD57^-$, $CD56^-$, granzyme B/

GB+, perforin−). Second, a significant number of freshly isolated TILs expressed CD57, but these cells were GB+, but mostly perforin−, a phenotype consistent with incompletely differentiated CTL. Third, CD56 expression was never found in freshly isolated $CD8^+$ TILs, but emerged in a subset of GB+ and perforin+ $CD8^+$ TILs after culture with interleukin (IL)-2/IL-15. CD56 and CD57 were never coexpressed, indicating that $CD8^+CD57^+$ and the $CD8^+CD56^+$ cells were unique T-cell lineages in TILs. Examination of the effector function found that it was the $CD8^+CD56^+$ subset that contained the most potent antitumor CTL population with the highest GB and perforin expression. In contrast, the $CD57^+$ subset has weak cytolytic, but high cytokine-secreting function. Moreover, the $CD57^+$ subset did not acquire potent tumor-killing activity, even after long-term culture with IL-2. Closer examination of this latter subset also found that a substantial fraction of $CD57^+$ cells expressed high levels CD27 and CD28 and were GB+, but perforin−. This atypical, incompletely differentiated, CTL population was not found in peripheral blood of melanoma patients or normal donors, indicating that it is unique to the tumor microenvironment. Functional analysis, however, found that the $CD8^+CD27^+CD28^+CD57^+$ subset was capable of further cell division and differentiation into perforin+ T cells after T-cell receptor triggering and culture with IL-2 suggesting that they are not terminally differentiated, but incompletely differentiated CTLs. These results indicate that $CD8^+CD57^+$ CTL differentiation in the melanoma microenvironment is impaired with an early extensive metabolizers phenotype incapable of potent antitumor killing activity. The appearance of the more cytolytic $CD8^+CD56^+$ subset may also be a key factor in the improved antitumor function of TILs used for adoptive T-cell therapy after extensive expansion ex vivo with IL-2.

Similar T-cell Receptor-β Sequences Identified in Melanoma-reactive T Cells and In Vivo 6-thioguanine-resistant T Cells From Melanoma Patients

*Cindy L. Zuleger**, Bret L. Bostwick†‡, Michael D. Macklin*, Qinglin Pei§, Michael A. Newton*§, Mark R. Albertini*†‖. **University of Wisconsin Carbone Comprehensive Cancer Center; †Medicine; §Statistics; ‡University of Wisconsin School of Medicine and Public Health; ‖William S. Middleton Memorial Veterans Hospital, Madison, WI.*

We studied clonally expanded in vivo mutant T cells from patients with advanced melanoma to investigate the in vivo T-cell responses to melanoma. Mutant hypoxanthine-guanine phosphoribosyltransferase-deficient T (MT) cells were obtained by 6-thioguanine (TG) selection from a melanoma patient's peripheral blood as clonal isolates or as mass cultures, and from tumor-bearing regional lymph nodes or distant melanoma sites as mass cultures. Wild-type T (WT) cells were obtained from all sites by analogous means without TG selection. cDNA sequences of the T-cell receptor-β chains (TRB) were used as unambiguous biomarkers of in vivo clonality and as indicators of T-cell specificity. We have reported enriched in vivo T-cell clonal amplifications, identified by identical TRB V-region, complementarity-determining region 3 (CDR3), and J-region gene sequences (TRB usage), among MT cells compared with WT cells earlier. The CDR3 is a hypervariable region implicated in T-cell specificity and antigen recognition. In addition, we identified common TRB usage by some MT cells within patients (ie, identical clonotypes in the blood and tumor) as well as between melanoma patients. To examine the potential specificity of expanded MT cells to melanoma, we constructed an empirical library of >700 TRB sequences from confirmed melanoma-reactive T cells from the primary literature. Data include T-cell specificity, human leukocyte antigen restriction, and TRB amino acid sequence. The empirical TRB library was compared with the in vivo MT and WT TRB sequences for 100% full, 6-amino acid, 5-amino acid, or 4-amino acid residues within the CDR3. Five of the 7 TRB sequences shared within or between melanoma patients were matched at the 100% 4-mer level, within the CDR3s, to CDR3s from the library. These 5 TRB sequences shared both within and between patients include sequences from MT clonal isolates (peripheral blood), MT mass cultures (peripheral blood and sites of tumor), and WT mass cultures (sites of tumor). Amino acid similarities were also found between the balance of the MT and WT dataset and the library. These results suggest that MT cells from melanoma patients contain T cells reactive to melanoma. Subsequent studies will functionally characterize MT cells to investigate this hypothesis. We conclude that MT cells from melanoma patients merit further study as probes into the immunobiology of melanoma.

Supported by a VA Merit Grant and gifts to the UWCCC.

MANIPULATION OF THE TUMOR MICROENVIRONMENT

Checkpoint Blockade in Tumor Immunotherapy: New Insights and Opportunities

James P. Allison. Memorial Sloan-Kettering Cancer Center, New York, NY.

It has become apparent that the effectiveness of active immunologic strategies for cancer therapy can be limited by cell intrinsic and extrinsic checkpoints that limit immune responses in order to maximize target destruction and minimize harm to normal tissues. The prototype of cell intrinsic "checkpoints" whose blockade enhances antitumor responses is cytotoxic T lymphocyte antigen (CTLA)-4, which has been extensively studied in animal models and shown to be quite effective in achieving complete tumor eradication and long-lasting tumor immunity. Over 4000 patients have been treated with an antibody to human CTLA-4 (Ipilimumab, Medarex, and Bristol-Meyers Squibb). Significant responses, including complete remissions, have been observed in about 15% of metastatic melanoma patients, with effects on survival in about 40% cases. This has led to considerable effort to identify biomarkers that would be useful in determining the impact of CTLA-4 blockade on immune responses to identify changes that correlate with clinical responses, as well as to inform combinatorial strategies that might enhance the effectiveness of Ipilimumab.

We have shown in melanoma and prostate cancer models in mice that tumor rejection is closely correlated with an increase in the ratio of both CD4 and CD8 effector cells to FoxP3+ regulatory cells. In chimeric mouse experiments, we have shown that the major target of CTLA-4 blockade are effector T cells. Blockade of CTLA-4 exclusively on T regulatory cells does not result in tumor rejection, while blockade of CTLA-4 on effector cells results in tumor rejection in about 50% of mice. However, blockade on both Treg and Teff is necessary for optimal tumor rejection.

In both the metastatic melanoma and prostate cancer patients, we have shown that existence of preexisting immune responses to tumor, as indicated by high titer of serum antibodies to the cancer testis antigen NY-ESO-1, is predictive of clinical benefit of Ipilimumab treatment. In a presurgical bladder cancer trial (Sharma et al), it was shown that Ipilimumab treatment results in an increase in the frequency of CD4 T cells that express high levels of the CD28/CTLA-4 homolog inducible T-cell co-stimulator (ICOS). We have shown that this is also true in metastatic melanoma and prostate cancer. In melanoma, sustained elevation of ICOS expression by CD4 T cells seems to correlate with clinical benefit. We have also shown that anti−CTLA-4 treatment is associated with elevation of the frequency of ICOShigh CD4 and CD8 T cells in mouse models, and that the size of transplantable tumors after the therapy is inversely correlated with the frequency of ICOShigh T cells. Finally, we have shown that the function of tumor-reactive T cells induced by anti−CTLA-4 treatment is impaired in ICOS-deficient mice. Together, these data indicate an important role for ICOS in the therapeutic effect of CTLA-4 blockade.

The Role of Indoleamine 2,3 Dioxygenase Enzymes (IDO-1 and IDO-2) in Prostate Cancer

Irina Banzola, Giovanni Sais, Cèdric Poyet, Tullio Sulser, Maurizio Provenzano. *Urology, University Hospital of Zurich, Zurich, Switzerland.*

Among the diverse tumor-derived factors involved in cell –contact-independent mechanism of tumor immune escape, indoleamine 2,3 dioxygenase (IDO) is a relevant one and its function is essential for the progression of malignancies. IDO is a cytosolic enzyme that catalyzes tryptophan degradation through the kynurenine pathway with an intermediate production of immunosuppressive catabolites. IDO-1 and IDO-2 are products of 2 different genes (INDO and the paralog INDOL1). Both proteins share 43% homology but those amino acids critical for the catalytic activity are well conserved. The 2 enzymes are constitutively expressed in a subset of human tissues, thus indicating a certain degree of functional redundancy. They can also be induced in response to inflammatory stimuli (interferon-γ, tumor necrosis factor-α), and activators such as lipopolysaccharide, although inflammation has a modest effect on IDO-2 compared with IDO-1. IDO was shown to be expressed in several cancers in which it regulates an important mechanism of immune escape. The enzyme exerts its immune suppression effects through the combination of tryptophan depletion to proliferative T cells and toxic activity through immunosuppressive catabolites, thus driving to cell growth arrest. We recently evaluated the expression of IDO-1 in prostate cancer (PCa) and benign prostate hyperplasia. The results from PCa patients showed the existence of 2 IDO-expressing groups: one with a lower IDO gene expression (IDO-low), comparable to IDO gene expression in benign prostate hyperplasia patients, and one with a higher IDO gene expression (IDO-high; $P < 0.0001$). In the IDO-high expressing patients, immunohistochemistry analysis showed the expression of IDO-1 exclusively in tumor cells. A significant correlation ($R^2 = 0.84$; $P = 0.0045$) between IDO-1 expression and its systemic impact (kynurenine/tryptophan ratio) was found. A relevant trend ($R^2 = 0.58$; $P = 0.13$) in relation to the clinical features of PCa patients was also seen. Testing the PCa cell lines, we identified different levels of expression of IDO-1 and IDO-2, though confirming the absence of IDO expression in normal prostate tissue. The relevant decrease of both IDO genes expression in bone, brain, and lymph node metastases, compared with localized tumor was notable ($\sim$250 folds). In particular, IDO-2 seems to be expressed in 2 isoforms: long (whole length enzyme) and short. When testing IDO-2 expression levels, the long isoform was only evident in localized primary prostate cancer ($\sim$400 folds), thus suggesting a different pattern of expression of both isoforms in local tumor and metastasis. IDO-1 and IDO-2 may have distinct functions within their tolerogenic role (due to either a different expression pattern or an enzyme-inhibitor specificity). Further insight into IDO pathway in PCa might be beneficial for both PCa diagnosis and treatment.

MUC1 Immunobiology in Transgenic Preclinical Models for Endometriosis and Endometrioid Ovarian Cancer

Raluca A. Budiu, Gina M. Mantia-Smaldone, Joan Brozick, Robert P. Edwards, Anda M. Vlad. *University of Pittsburgh School of Medicine, Pittsburgh, PA.*

Ovarian epithelial tumors are the leading cause of death from gynecologic malignancies and endometriosis is the only currently described precursor lesion. MUC1 mucin is a tumor-associated antigen and a potential target for cancer immunotherapy. Human and murine mucin 1 share little homology in the extracellular region and transgenic mouse models with de novo lesions expressing the human antigen are needed.

We proposed here to test the MUC1 immunogenicity in both malignant and premalignant ovarian lesions. To study MUC1 changes during disease progression and in vivo immune regulation in endometriosis and ovarian cancer, we crossed the MUC1 transgenic mice, which express human MUC1 under the endogenous promoter with the previously described KrasG12D/+ and KrasG12D/+PtenloxP/loxP mice, respectively. MUC1+/−KrasG12D/+ mice progress to endometriosis-like lesions. The benign lesions show typical glandular morphology and are positives for MUC1, estrogen receptor, and cytokeratin-7, markers classically used to diagnose endometriosis. High titers of MUC1-specific antibodies were detected and the percentages of FOXP3-positive cells (T regulatory cell) in paraaortic lymph nodes of mice with ovarian endometriotic lesions were increased. The double transgenic KrasPten mice progress to metastatic ovarian tumors accompanied by ascites. The tumors have endometrioid morphology and show increased phosphorylation of Akt, PI3K, and MEK, increased vascularization, and abundant T-cell infiltrates. We identified increased accumulation of T regulatory cell in the paraaortic (draining) lymph nodes and the presence of Th17 cells in ascites.

Experiments in triple transgenic MUC1+/−KrasG12D/+PtenloxP/loxP are in progress. Our results reveal important immune regulatory mechanisms in mice with ovarian endometriosis and ovarian tumors and provide unique opportunities for future in vivo testing of MUC1 vaccines.

Myeloid-derived Suppressor Cells and Tumor Microenvironment

Dmitry I. Gabrilovich. H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL.

Myeloid-derived suppressor cells (MDSCs) represent an intrinsic part of myeloid cell lineage and comprise myeloid progenitors and precursors of myeloid cells. In a healthy host, upon generation in bone marrow immature myeloid cells (IMC), they quickly differentiate into mature granulocytes, macrophages, or dendritic cells. Cancer increased the production of IMC that is associated with partial block of their differentiation and most importantly pathologic activation of these cells manifests in upregulation of arginase, inducible nitric oxide synthase (iNOS) and NO production, and increased level of reactive oxygen species (ROS). This results in the expansion of IMC with immune suppressive activity. Accumulation of MDSC was detected in practically all mouse tumor models and in patients with different types of cancer. In mice, MDSCs are characterized by the coexpression of myeloid lineage differentiation antigen, Gr1 and CD11b. In humans, MDSCs are currently defined as $CD14^-CD11b^+$ cells or more narrowly as cells that express the common myeloid marker, CD33, but lack the expression of markers of mature myeloid and lymphoid cells and the major histocompatibility complex class II molecule human leukocyte antigen-DR. It appears that MDSC in peripheral lymphoid organs and in tumor sites use different mechanisms of T-cell suppression. In peripheral lymphoid organs, MDSCs have high level of ROS and relatively low levels of NO and arginase. MDSCs cause antigen-specific tolerance of $CD8^+$ T cells. We tried to identify the mechanism of MDSC-mediated T-cell tolerance. Using double T-cell receptor (TCR) $CD8^+$ T cells, we have demonstrated that MDSCs induced tolerance against only the peptide, which was directly presented by these cells. This effect was associated with nitration of the molecules on the surface of $CD8^+$ T cells localized to the site of physical interaction between MDSC and T cells. After incubation with MDSCs, only nitrotyrosine-positive $CD8^+$ T cells demonstrated profound nonresponsiveness to the specific peptide, whereas nitrotyrosine-negative $CD8^+$ T cells responded normally to that stimulation. Incubation of antigen-specific $CD8^+$ T cells with peptide-loaded MDSC did not induce downstream signaling of TCR. However, it prevented subsequent signaling from peptide-loaded dendritic cells. MDSCs caused dissociation between TCR and CD3ζ molecules disrupting TCR complexes on T cells. In contrast, MDSCs from tumor sites have relatively low ROS production but high levels of NO and arginase. As a result, MDSC inhibited T-cell function in an antigen-independent manner. We have demonstrated that in the tumor microenvironment, splenic MDSCs rapidly upregulate iNOS and arginase and are rapidly converted to antigen nonspecific

suppressor cells. MDSCs quickly differentiate into tumor-associated macrophages with potent T-cell suppressive activity. The potential mechanisms of this phenomenon are discussed.

Alpha-Tocopheryloxyacetic Acid: A Tumor Apoptogen That Stimulates the Antitumor Immune Response

*Tobias Hahn**, Yuhuan Li†, Kendra Garrison*, Emmanuel T. Akporiaye*. **Tumor Immunology and Therapeutics; †Cancer Immunobiology, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR.*

Alpha-tocopheryloxyacetic acid (α-TEA) is a novel ether derivative of α-tocopherol, which has generated interest as a potential chemotherapeutic agent because of its selective toxicity toward tumor cells and its ability to suppress tumor growth in various rodent and human xenograft models. We recently reported that when supplied orally in the diet, α-TEA inhibited the growth of both a transplanted (4T1) and a spontaneous (MMTV-PyMT) mouse model of breast cancer. Although several studies have implicated the induction of proapoptotic signaling pathways as the primary mechanism of α-TEA – mediated tumor cell death, we have shown that α-TEA synergizes with dendritic cell (DC) vaccines to inhibit murine tumor growth. As little is known about the possible immunologic mechanisms underlying the in vivo α-TEA effects, we evaluated the contribution of the adaptive immune response to the α-TEA antitumor effect by depletion of T cells. Whereas α-TEA caused a 54% reduction in tumor size and 10-day prolongation of survival in T-cell-sufficient mice, simultaneous depletion of $CD4^+$ and $CD8^+$ T cells decreased the α-TEA efficacy to 35% tumor reduction and no survival advantage. To determine the effect of α-TEA on DCs that are crucial for an effective T-cell antitumor response, DCs were coincubated with supernatant generated by treating tumor cells with α-TEA (α-TAGS). α-TAGS treatment led to the upregulation of costimulatory molecules and interleukin-12 (IL-12p70) secretion by DCs. Immunization with α-TAGS – matured DCs (α-TAGS DC), induced higher interferon-γ secretion in mice than nonmatured DCs, suggesting that α-TAGS DCs have an increased ability to prime T cells. To examine this possibility, DCs were pulsed with α-TAGS from tumor cells expressing the OVA model antigen and coincubated with OVA-specific OT-I $CD8^+$ T cells, which resulted in vigorous OT-I T-cell proliferation, indicative of enhanced cross-presentation. Recently, tumor cell autophagosomes have been implicated as antigen carriers for cross-presentation. Therefore, we examined α-TAGS for the definitive autophagy marker LC3 II, which was increased in α-TAGS in comparison with untreated cells. These data suggest that α-TEA treatment generates tumor antigen-containing autophagosomes that can be efficiently cross-presented by DCs to $CD8^+$ T cells and that the T cell-mediated antitumor immune response may be partially responsible for α-TEA – mediated tumor growth suppression. These studies may shed light on the mechanisms of action of α-TEA and may prove useful in designing immune-stimulating strategies to boost the antitumor effects of α-TEA in cancer patients.

Indoleamine 2,3-dioxygenase Expression by Murine T Cells After In Vitro Exposure to Tumor Culture Supernatant

*Tyce Kearl**, Bryon Johnson*,†,‡. **Microbiology and Molecular Genetics; †Pediatrics, Medical College of Wisconsin; ‡Children's Research Institute, Children's Hospital of Wisconsin, Milwaukee, WI.*

Functional suppression of tumor-reactive T cells occurs by a variety of means and is a major obstacle to effective treatment of cancer by immunotherapy. Expression of indoleamine 2,3-dioxygenase (IDO) by many types of tumors and by dendritic cells is a well-known mechanism of T-cell suppression in the tumor microenvironment and in tumor-draining lymph nodes. IDO mediates T-cell suppression through the production of toxic metabolites and by depleting the local environment of tryptophan, thereby activating the GCN2 kinase pathway. Expression of IDO by T cells themselves has only been noted in the literature a few times, and the functional significance of T-cell – expressed IDO is unclear. We have identified several tumor cell lines that induce IDO expression in murine T cells. IDO mRNA is upregulated in T cells after exposure to culture supernatant from both U2OS human osteosarcoma cells engineered to overexpress murine macrophage migration inhibitory factor (MIF) and AGN2a murine neuroblastoma cells that constitutively produce high amounts of MIF, but not after exposure to supernatant from tumor cells that produce low levels of MIF. IDO upregulation was detected in both purified $CD4^+$ and $CD8^+$ T cells. IDO-expressing T cells concomitantly displayed decreased proliferation in response to activation, as detected in H_3-thymidine uptake assays and by CFSE staining. These findings provide the opportunity to further elucidate the functional significance of IDO expression in T cells. The AGN2a neuroblastoma tumor model is being used to determine whether adoptive transfer of IDO $-/-$ T cells after myeloablative total body irradiation can affect tumor clearance and T-cell function. These findings also suggest that MIF plays a direct or indirect role in inducing IDO expression in murine T cells.

Cellular and Molecular Requirements for Rejection of B16 Melanoma in the Setting of Regulatory T-cell Depletion and Homeostatic Proliferation

Justin Kline, Long Zhang, Thomas Gajewski. *Medicine, University of Chicago, Chicago, IL.*

We have recently demonstrated that adoptive transfer of T regulatory-depleted T cells into lymphopenic mice can result in potent rejection of B16 melanoma in vivo. In this study, we have investigated the cellular and molecular requirements for tumor rejection by CD25-depleted splenic T cells in $RAG^{-/-}$ or irradiated C57BL/6 recipient mice. Using donor T cells from $CD4^{-/-}$ or $CD8^{-/-}$ mice, we observed that both $CD4^+$ and $CD8^+$ T cells were necessary for B16.SIY rejection. The contribution of conventional $CD4^+$ T cells led us to investigate the mechanisms of T-cell help. Interestingly, tumor rejection still occurred in irradiated $CD40^{-/-}$ hosts, and also with transfer of interleukin-$2^{-/-}$ $CD4^+CD25^-$ T cells along with wild-type $CD8^+$ T cells into $RAG2^{-/-}$ hosts. These results indicate that the role of $CD4^+$ T cells is not via provision of CD40L to antigen-presenting cells and not through the provision of interleukin-2 to $CD8^+$ T cells. Consistent with the tremendous expansion of antigen-specific $CD8^+$ T cells observed, we found that host tumor antigen cross-presentation was required, as tumor rejection did not occur in irradiated $Kb^{-/-}$ or $B7.1/B7.2^{-/-}$ mice after CD25-depleted T-cell transfer. At the effector phase, host interferon (IFN)-γ production, and both, the production of and sensitivity to IFN-γ by adoptively transferred T cells, were necessary for the rejection of B16.SIY. Finally, production of tumor necrosis factor-α and perforin by adoptively transferred CD25-depleted T cells was dispensable. Collectively, these results support a model in which host tumor antigen cross-presentation and B7 costimulation to $CD8^+$ T cells, along with an undefined role of $CD4^+$ T cells, generate effector cells that depend at least upon IFN-γ for tumor control. The elimination of T regulatory cells and homeostatic proliferation maintain persistent T-cell function in the context of a growing tumor. This straightforward adoptive transfer strategy has strong potential for translation to the clinic.

Generation of Antigen-presenting Cells From Tumor-infiltrated CD11B Myeloid Cells With DNA Demethylating Agent 5-AZA-2′-deoxycytidine

Sergei Kusmartsev, Irina Daurkin, Evgeniy Eruslanov, Johannes Vieweg. *University of Florida, Gainesville, FL.*

Tumor-recruited CD11b myeloid cells, including myeloid-derived suppressor cells, play a significant role in tumor progression, as these cells are involved in tumor-induced immune suppression and tumor neovasculogenesis. On the other hand, the tumor-infiltrated CD11b myeloid cells could potentially be a source of immunostimulatory antigen-presenting cells (APCs), as most of these cells represent common precursors of both dendritic cells and macrophages. Here we investigated the possibility of generating mature APCs from tumor-infiltrated CD11b myeloid cells. We demonstrate that in vitro exposure of freshly excised mouse tumors to DNA methyltransferase inhibitor 5-AZA-2′-deoxycytidine (decitabine, AZA) results in the selective elimination of tumor cells, but unexpectedly, it also enriches $CD45^+$ tumor-infiltrated cells. The majority of "post-AZA" surviving $CD45^+$ tumor-infiltrated cells was represented by CD11b myeloid cells. A culture of isolated tumor-infiltrated CD11b cells in the presence of AZA and granulocyte macrophage-colony-stimulating factor promoted their differentiation into mature F4/80/CD11c/major histocompatibility complex class II-positive APCs. These tumor-derived myeloid APCs produced substantially reduced amounts of immunosuppressive [interleukin (IL)-13, IL-10, prostaglandin E_2), proangiogenic (vascular endothelial growth factor, matrix metalloproteinase-9) and proinflammatory (IL-1β, IL-6, macrophage inflammatory protein-2) mediators than their precursors, freshly isolated tumor-infiltrated CD11b cells. Vaccinating naive mice with ex vivo-generated tumor-derived APCs resulted in the protection of 70% mice from tumor outgrowth. Importantly, no loading of tumor-derived APCs with exogenous antigen was needed to stimulate T-cell response and induce the antitumor effect. Collectively, our results for the first time demonstrated that tumor-infiltrated CD11b myeloid cells can be enriched and differentiated in the presence of DNA demethylating agent, 5-AZA-2′-deoxycytidine, into mature tumor-derived APCs, which could be used for cancer immunotherapy.

The Roles of COX-2 Inhibitor in Regulatory T Cells and Th17 Cells' Tumor Immune Regulation

Sung Yong Lee, Hye Ok Kim, Hye Kyoung Choi, Kyoung Ju Lee, Gyu Young Hur, Jae Jeong Shim, Kwang Ho In, Kyoung Ho Kang, Se Hwa Yoo. *Department of Internal Medicine, Korea University Medical Center, Seoul, South Korea.*

Background: COX-2 and its metabolite, prostaglandin (PG)E_2, affect tumorigenesis and tumor-induced immune suppression. In the field of tumor immunology, regulatory T cells (Tregs) and Th17 cells are emerging as important targets. The presence of Tregs might be important for inducing T-cell suppression and thus allowing tumor growth. Th17 cells play an active role in inflammation and autoimmune diseases. These cell types have reciprocal roles in inflammatory conditions. However, very little is known about the roles of the COX-2 inhibitor in Tregs and Th17 cells in tumor development. Therefore, we investigated the effects of COX-2 inhibitors on Treg and Th17 cell activation in a tumor model.

Methods: In an in vitro assay, to evaluate the effects of COX-2 on the proliferation of Tregs and Th17 cells, $CD4^+CD62L^+$-naive T cells were incubated in the presence of transforming growth factor (TGF)-β, with or without interleukin (IL)-6, and PGE_2 or a COX-2 inhibitor (celecoxib) was then added. Through western blotting, we assessed Foxp3 and ROR-γ, which were recently described as being essential for the differentiation of Tregs and Th17 cells, respectively. In the in vivo assay, 20 mice were randomized into a group of normal control, Lewis lung cancer cells (3LL)-inoculated control, and celecoxib (10 or 100 mg/kg/d)-treated 3LL-inoculated mice groups. The tumor mass and spleen of each mouse were removed for isolation of splenocytes and tumor-infiltrating lymphocytes for fluorescence-activated cell sorting analysis, real-time polymerase chain reaction, and western blotting.

Results: When $CD4^+CD62L^+$-naive T cells were stimulated under the Treg-promoting condition (TFG-β only), the expression of Foxp3 increased. When naive T cells were stimulated under Th17-promoting conditions, TGF-β and IL-6 induced significant ROR-γ expression and IL-17 secretion. When naive T cells were treated with PGE_2 and TGF-β, the expression of Foxp3 increased. These increased expressions were decreased in the presence of celecoxib. We assessed the effects of COX-2 on IL-17 production in TGF-β – stimulated and IL-6 – stimulated naive T cells. We found that IL-17 production was increased with PGE_2 and decreased with celecoxib treatment, in a dose-dependent manner. The expressions of Foxp3 and ROR-γ in the tumor mass were decreased in the celecoxib-treated mice groups. Fluorescence-activated cell sorting analysis demonstrated a decline in the percentage of $CD4^+IL17+$ and $CD4^+CD25^+$ in the celecoxib-treated groups.

Conclusions: The results of this study show that PGE_2 increases the differentiation of Tregs and Th17 cells, whereas a COX-2 inhibitor inhibits the differentiation of both Tregs and Th17 cells. Although Tregs and Th17 cells have reciprocal roles, the COX-2 inhibitor decreases the differentiation of both Treg and Th17 cells. This mode of immunoregulatory action by a COX-2 inhibitor requires further investigation.

Ethyl Pyruvate Administration Inhibits Hepatic Metastases and Alters Tumor Cell Metabolism

*Xiaoyan Liang**,†, Antonio Romo-de Vivar*,†, Tao Wang†, Nicole E. Schapiro*, Bennett Van Houten*, Andrew A. Amoscato*, Herbert J. Zeh*, Michael T. Lotze*, Michael E. de Vera*,†. **Surgery, Transplantation Institute;* †*Surgery, Hillman Cancer Center, Pittsburgh, PA.*

Pyruvate is the endproduct of glycolysis and the starting substrate for the tricarboxylic acid cycle, which generates NADH and ATP during oxidative phosphorylation. Ethyl pyruvate (EP), the ethyl ester of pyruvate, improves survival and organ dysfunction in inflammatory animal models of sepsisand ischemia reperfusion. The mechanism of the EP biologic function is unclear. We have shown that EP has antitumor activity in a murine liver metastasis model. Pretreatment with EP (40 or 80 mg/kg intraperitoneal) 30 minutes before infusion of tumor cells and continuing daily for 9 days significantly inhibited tumor growth in a dose-dependent fashion, with 80 mg/kg EP achieving $> 70\%$ reduction in the number of tumor nodules when compared with untreated animals (Table 1). TUNEL staining demonstrated an increase in tumor apoptosis in mice treated with EP, and tumor cells treated with EP in vitro had marked increases of both LC3-II and cleaved PARP, consistent with both enhanced autophagic flux and apoptosis. According to the Warburg hypothesis, increased conversion of glucose to lactic acid associated with decreased mitochondrial respiration is a unique feature of tumors. In this study, we hypothesized that EP alters tumor cell metabolism. We analyzed that the bioenergetic phenotype of tumor cells was by measuring cellular respiration and glycolysis rate using a novel real-time assessment of oxidative phosphorylation and glycolysis.

Methods: In all, 4×10^4 cells/well of MC38 and PANCO2 tumor cells were seeded into the XF24 V7 microplate. Cells were pretreated with/without EP (5 and 10 mM) for 60 minutes before measurement. The XF24 Analyzer was used with XF Assay Kits (Seahorse Bioscience) to measure extracellular flux changes in oxygen consumption rate (OCR) and the rate of proton excretion in the media (extracellular acidification rate, ECAR).

Results: MC38 and PANCO2 tumor cells had substantially different bioenergetic phenotypes. MC38 cells had lower basal OCR (Graph) and higher basal ECAR than PANCO2 cells (56 ± 6 vs. 8 ± 2, respectively, $P < 0.05$). Use of 2-deoxyglucose to block glycolysis significantly enhanced OCR in PANCO2 from 437 ± 50 to 1486 ± 154 pmoles/min but not in MC38, suggesting that MC38 cells are more dependent on glycolysis. EP enhanced cellular respiration (OCR) in MC38 and decreased basal ECAR significantly without observable changes in PANCO2 cells.

TABLE 1. (Liang)

	MC 38	PANCO2
UT control	85 ± 9	1486 ± 154
EP 5 mM	347 ± 7	1368 ± 14
EP 10 mM	138 ± 29	1496 ± 204

Summary: These findings demonstrate that EP alters MC38 tumor cell metabolism significantly. These metabolic effects may be related to the antitumor activity of EP.

Cyclic Pifithrin-α Decreases High-mobility Group Box-1 Expression and Increases Cell Viability, ATP Conservation, HMGB1 Translocation, and Autophagy

Kristen M. Livesey, Daolin Tang, Patricia Loughran, William Buchsler, Michael T. Lotze. *Surgery, University of Pittsburgh, Pittsburgh, PA.*

Background: Autophagy, literally self-eating, is an important mechanism in which eukaryotic cells respond to stress and remove dysfunctional proteins and organelles. Autophagy in tumors and virally infected cells is important for antigen cross-presentation by dendritic cells. In tumors, autophagy has a role in tumorigenesis and tumor progression. Inhibiting autophagy can promote carcinogenesis by increasing the levels of protein synthesis, allowing mutations to accumulate. Inhibiting autophagy can enhance retention of damaged organelles. In response to hypoxia, acidosis, or nutrient deprivation, autophagy is accelerated in cancer cells in the later stages of tumor progression. Autophagy is downregulated in breast cancer, prostate cancer, and upregulated in pancreatic cancer and colon cancer. High-mobility group box-1 (HMGB1) plays a critical role in tumors as it inhibits apoptosis, promotes autophagy, and enhances cancer survival. HMGB1 is a nuclear protein in unstressed cells but translocates to the cytosol in stressed cells. Mediators of this translocation and the cytosolic role of HMGB1 are undefined. It was recently discovered that p53, the most commonly mutated gene in cancer, has a cytosolic role in inhibiting autophagy, making this protein of interest in the possible regulation of HMGB1.

Methods: To determine whether p53 regulates HMGB1, we evaluated the effects of cyclic pifithrin-α (PFT-α), a pharmacologic antagonist initially believed to inhibit p53, on apoptosis and autophagy induced by rapamycin and starvation (HBSS) in wild-type (wt) and HMGB1 (KO) mouse embryonic fibroblasts (MEFs). The mechanism of action of PFT-α is currently under debate with cyclin D1 as a proposed target.

Wt and KO MEFs were treated with PFT-α, HBSS, rapamcyin, PFT-α pretreatment + HBSS, and PFT-α pretreatment + rapamcyin. Cell viability, ATP release and HMGB1, p53, cleaved PARP, Beclin-1, and LC3-I/II (autophagy markers) expression were quantified. HMGB1, LC3, and Beclin-1 were quantified and localized by imaging cytometry. Colocalization of autophagosomes and mitochondria, lysosomes, and endoplasmic reticulum was determined by confocal microscopy.

Results/Conclusions: PFT-α decreases the expression of HMGB1 and increases cell viability, ATP conservation, HMGB1 translocation, and autophagy. p53 increases HMGB1 expression and KO MEFs have decreased autophagy and increased apoptosis. This suggests that p53 and HMGB1 interactions regulate the balance between autophagy and apoptosis. We are now exploring the mechanism by which cytosolic HMGB1 and p53 interact and regulate the consequences of enhanced cell death or autophagy, thereby driving the tumor microenvironment and the resultant host response.

Eosinophilic Granulocytes Modulate Tumor Microenvironment by Oxidizing Damage-associated Molecular Pattern Molecules Derived From Necrotic Tumor Cells

*Ramin Lotfi**†, Gloria I. Herzog*, Richard A. DeMarco†, Donna Beer-Stolz‡, James J. Lee§, Anna Rubartelli‖, Hubert Schrezenmeier*, Michael T. Lotze†. **Institute of Transfusion Medicine, University of Ulm, Ulm, Germany;* ‖*Laboratory of Cell Biology, National Cancer Research Institute, Genova, Italy;* †*Hillmann Cancer Center;* ‡*Department of Cell Biology and Physiology, Center for Biologic Imaging, University of Pittsburgh, Pittsburgh, PA;* §*Division of Pulmonary Medicine, SCJMRB-Research, Mayo Clinic Arizona, Scottsdale, AZ.*

Eosinophilic granulocytes (Eos) are found in increased numbers within the necrotic areas of tumors. Necrotic death is associated with release of damage-associated molecular pattern molecules (DAMPs), which influence the tumor microenvironment enhancing proliferation of adjacent cells, angiogenesis, and inflammation. We show that necrotic material from tumor or mesenchymal stem cell lysates containing DAMPs induce Eos degranulation (release of major basic protein and eosinophil peroxidase) and enhance Eos oxidative burst whereas the stimulatory capacity of cell lysates is significantly diminished after oxidation. High mobility group box 1 (HMGB1), a prototypic DAMP, released after necrosis, but not apoptosis, induced a similar effect on Eos. In addition, we demonstrate that HMGB1 enhances Eos survival and acts as a chemoattractant. Consistently, we show that Eos expresses an HMGB1 receptor, the receptor for advanced glycation endproduct (RAGE), and that anti-RAGE could diminish the HMGB1-mediated effects. Of all the tested biologic activities, Eos responds most sensitively to the presence of necrotic material including HMGB1 with the generation of oxidants. We postulate that Eos "sense" stressed cells, migrating to and responding to areas of tissue injury/necrosis. Oxidation of cell lysates reduces their biologic activity when compared with native lysates. We postulate that Eos-associated modulation of immunity within tumor and other damaged tissues may be primarily by promoting oxidative degradation of DAMPs. Novel therapeutic strategies may be considered by advancing oxidative denaturation of released necrotic material using Eos or other aerobic strategies.

Antiviral State Segregates 2 Molecular Phenotypes of Pancreatic Adenocarcinoma: Potential Relevance for Adenoviral Gene Therapy

*Vladia Monsurrò**, S. Beghelli*, Richard Wang†, S. Barbi*, S. Coin*, G. Di Pasquale‡, S. Bersani*, M. Castellucci*, C. Sorio*, S. Eleuteri*, Andrea Worschech†, J. Chiorini‡, P. Pederzoli§, H. Alter†, Francesco Marincola†, A. Scarpa*. *Departments of* **Pathology;* §*Surgical and Gastroenterological Sciences, University of Verona Medical School, Verona, Italy;* †*Department of Transfusion Medicine, and Center for Human Immunology (CHI);* ‡*NIDCR/Gene Therapy and Therapeutics Branch (NIDCR), National Institutes of Health, Bethesda, MD.*

Pancreatic adenocarcinoma (PDAC) remains a leading cause of cancer mortality for which novel gene therapy approaches relying on tumor-tropic viruses are being tested.

Applying global transcriptional profiling to PDAC samples derived from primary cancers, we observed 2 clearly distinguishable phenotypes according to the expression of interferon (IFN)-stimulated genes (ISGs). The 2 PDAC phenotypes could be readily recognized by the detection of the Myxovirus-resistance A (MxA) protein, whose expression best represents the downstream activation of IFN-dependent pathways. The 2 molecular phenotypes discovered in primary PDACs were also observed among established PDAC cell lines, suggesting that these phenotypes are an intrinsic characteristic of cancer cells independent of their interaction with the host's microenvironment. To test whether ISG expression in PDACs confers antiviral activity, we transduced PDAC cell lines with green fluorescent protein or luciferase-expressing adenovirus and observed a significant delay in the kinetics of reporter genes expression in ISG-expressing cell lines suggesting a potential resistance to adenoviral infection. Similar results were observed when cells were transduced with Adeno-associated viruses 5 and 6. Even more, the cell lines bearing the MxA-positive phenotype are protected from the infection and lysis mediated by the Adeno5 wild-type virus.

Our study identified 2 molecular phenotypes of PDAC, characterized by an overexpression of downstream IFN-related genes and easily recognized by the expression of the MxA protein. The 2 phenotypes are characterized by different permissivity to viral vectors used for gene therapy and susceptibility to viral lysis. We suggest that their recognition might help in the selection of patients enrolled in virally mediated gene therapy trials.

Tumor-selective Induction of Cytotoxic T Lymphocyte-attracting Chemokines and Local Suppression of T Regulatory Cell-attracting Chemokines by the Combination of Interferon-α, Poly-I:C, and COX Inhibitors

*Ravikumar Muthuswamy**†, Beth J. Fallert‡, Erik Berk*, The Minh Luong§, James Schlesselmann§, Herbert Zeh*†, Todd Reinhart‡, David Bartlett*†, Pawel Kalinski*†‡. **Surgery; ‡Infectious Diseases and Microbiology; §Statistics; †University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA.*

Background: Local infiltration with $CD8^+$ effector T cells [cytotoxic T lymphocytes (CTLs)] can predict long-term survival of patients with colorectal cancer (CRC), indicating the key role for the ability of CTLs to enter tumor tissues in the effectiveness of cancer immunity. In contrast, tumor infiltration with T regulatory (Treg) cells predicts poor outcomes. In this study, we determined the feasibility of selectively promoting CTLs into tumors, by enhancing the production of CTL-attracting chemokines within the tumor tissues, with concomitant suppression of Treg-attracting chemokines.

Materials and Methods: Correlation between various T-cell markers and chemokines in tumor tissues was determined by real-time reverse transcription-quantitative polymerase chain reaction analysis (Taqman). Chemokine expression in untreated tumors and ex vivo-cultured tumor explants exposed to different modulatory agents, was analyzed by Taqman and enzyme-linked immunosorbent assay. The ability of CTLs and Tregs to migrate toward the differentially treated tumors was tested in chemotaxis chambers.

Results: We observed a strong positive correlation between the expression of several CTL markers within the metastatic CRC specimen and the local expression of CCL5 and CXCL10. Treatment of tumor tissues with COX-2 inhibitors, combined with interferon (IFN)-α and toll-like receptor-3 ligand, selectively enhanced Teff cells attracting IP10 and RANTES and down-regulated undesired Treg-attracting CCL22 within the CRC lesions. Moreover, the combined treatment selectively enhanced the CTL-attracting chemokines in tumor tissues rather than in marginal healthy tissues, with most of the chemokines being produced by tumor-infiltrating antigen-presenting cells. In accordance with the differential ability of Teff and Treg cells to respond to different sets of chemokines, we observed that the COX inhibitor/IFN-α treatment increased the ability of tumors to attract effector-type $CD8^+$ T cells with a concomitant reduction of Treg-attracting function.

Conclusions: Our data indicate the possibility to correct the undesirable, Treg-favoring chemokine profiles in tumor tissues, using the defined combination of IFNs, toll-like receptor-Ls, and inhibitors of prostaglandin synthesis. Our upcoming clinical trials will determine whether such tumor-selective chemokine modulation can enhance the clinical effectiveness of therapeutic vaccines against CRC (and melanoma), by directing the vaccination-induced CTLs to tumors and limiting local Treg attraction.

Supported by 1PO CA132714.

Depletion of Macrophages in the Tumor Microenvironment Using Liposomal Clodronate Augments Adoptive T-cell Therapy in a Melanoma Mouse Model

Vijay Peddareddigari, Weiyi Peng, Alexander Gelbard, Patrick Hwu, Willem W. Overwijk. *Melanoma Medical Oncology, U.T.M.D. Anderson Cancer Center, Houston, TX.*

Background: Tumor-associated macrophages (TAMs) are known to promote the growth of solid tumors such as melanoma. They can also contribute to suppression of T-cell – mediated antitumor immune response. We targeted TAMs for depletion using clodronate liposomes (CLs) as a potential therapeutic application to augment adoptive T-cell therapy (ACT) in a B16 melanoma mouse model.

Methods: The effect of CL or plain control liposome (PL) on B16 tumor cells, macrophages, and other leukocytes was evaluated in vitro using XTT assay and flow cytometry. An in vitro coculture system involving T cells, macrophages, and B16 tumor cells was developed to evaluate the effect of macrophages preconditioned with B16 melanoma-conditioned media on interferon-γ production by pmel-1 T cells recognizing the gp100 melanoma antigen. The effect of systemic macrophage depletion on therapeutic efficacy of ACT was studied.

Results: CL was selectively toxic to macrophages both in vitro and in vivo but was nontoxic to B16 melanoma cells and T cells. Macrophages pretreated with B16 tumor cell-conditioned media effectively suppressed tumor-specific interferon-γ production by melanoma-specific gp100 pmel-1 T cells demonstrating a mechanism of tumor immunosuppression.

In vivo, CL administration decreased B16 melanoma growth by 70%. Analysis of the tumor tissue showed that the population of F4/80+ macrophages decreased from 7.4% ± 0.7% to 2.7% ± 0.5% whereas that of $CD8^+$ T cells increased from 3.3% ± 0.5% to 7.6% ± 0.9% when treated with PL or CL, respectively. ACT using gp100 pmel-1 T cells alone resulted in 67% decrease in tumor growth but ACT after CL administration resulted in 92% decrease in tumor growth including 62% complete regression in a setting of minimal tumor burden and 30% when palpable tumors were treated.

Conclusions: TAMs can suppress antitumor T-cell responses and their depletion from the microenvironment is a novel approach to augmenting ACT for melanoma.

Opposing Roles for HIF-1α and HIF-2α in Tumor-infiltrating Macrophages

Julie M. Roda, Timothy D. Eubank, Clay B. Marsh. *Internal Medicine, The Ohio State University, Columbus, OH.*

Macrophage secretion of vascular endothelial growth factor (VEGF) in response to the hypoxic tumor microenvironment is well known to contribute to tumor growth and metastasis. We have previously demonstrated that macrophages stimulated with granulocyte macrophage-colony-stimulating factor (GM-CSF) at low O_2 levels also secrete high levels of a soluble form of the VEGF receptor (sVEGFR-1) that neutralizes VEGF and inhibits its biologic activity. Here we investigate the role of the hypoxia-inducible factor (HIFs) in macrophage production of sVEGFR-1. Experiments using macrophages in which HIF-1α or HIF-2α was neutralized with a specific siRNA demonstrated that sVEGFR-1 production was dependent on HIF-α, whereas VEGF production required HIF-1α. These findings were confirmed in a murine model in which the growth of B16F10 melanomas was evaluated in mice with a macrophage selective deletion of HIF-1α or HIF-2α (LysMcreHIF-1α or LysMcreHIF-2α mice). GM-CSF reduced microvessel density and inhibited tumor growth in Wild type (WT) mice, an effect that was dependent on sVEGFR-1 production. GM-CSF inhibited tumor growth to a greater extent in LysMcreHIF-1α mice than in WT mice, but did not inhibit tumor growth in LysMcreHIF-2α mice. GM-CSF treatment of LysMcreHIF-1α mice resulted in a greater reduction in tumor angiogenesis as compared with WT mice, but no decrease in tumor angiogenesis was observed in response to GM-CSF in LysMcreHIF-2α mice. Real-time polymerase chain reaction performed on purified tumor macrophages revealed that macrophages within the tumors of LysMcreHIF-1α mice treated with GM-CSF produced less VEGF but comparable amounts of sVEGFR-1 as compared with macrophages from tumors of WT mice. In contrast, tumor-infiltrating macrophages from GM-CSF – treated LysMcreHIF-2α

mice produced less sVEGFR-1 than macrophages from tumors of WT mice, with no difference in VEGF levels. Taken together, these findings suggest a model in which GM-CSF treatment induces sVEGFR-1 production from tumor-associated macrophages, inhibiting angiogenesis and reducing tumor growth. In mice with HIF-1α – deficient macrophages, GM-CSF induces sVEGFR-1 production but VEGF production is inhibited, resulting in a net decrease in angiogenesis and tumor growth. In mice with HIF-2α – deficient macrophages, GM-CSF fails to induce sVEGFR-1 production but VEGF production is preserved, resulting in increased angiogenesis and accelerated tumor growth. Therefore, although hypoxia is generally considered to be a proangiogenic state, our results demonstrate a significant antiangiogenic effect of hypoxia in a GM-CSF – rich environment. Furthermore, these results demonstrate opposing roles for the HIFs in tumor angiogenesis, with HIF-1α exhibiting proangiogenic behavior via its effects on VEGF secretion, whereas HIF-2α exhibits antiangiogenic behavior by inducing the production of the endogenous angiogenesis inhibitor, sVEGFR-1.

Manipulation of the Tumor Microenvironment by Cytotoxic T Lymphocyte Antigen-4 Blockade

Padmanee Sharma. M. D. Anderson Cancer Center, Houston, TX.

Biomarker studies used with immunotherapeutic strategies in the clinic have typically involved monitoring immunologic changes within systemic circulation; however, recent data indicate that immunologic changes within tumor tissues will be more likely to predict clinical responses. In several murine models, blockade of the T-cell inhibitory molecule, cytotoxic T lymphocyte antigen (CTLA)-4, has been shown to result in tumor rejection, which correlates with an increase in the ratio of effector-to-regulatory T cells. More than 4000 cancer patients have now been treated with anti – CTLA-4 antibody on clinical trials, but there have been limited data correlating immunologic changes with clinical outcomes for 2 major reasons: (1) most clinical trials are conducted in the metastatic disease setting, which makes it difficult to access tumor tissues for immunologic studies and (2) a marker to define effector T cells in cancer patients treated with anti – CTLA-4 therapy has not been established. To obtain such data we conducted the first presurgical clinical trial with anti – CTLA-4 antibody in a cohort of patients with localized bladder cancer.

Our trial focused on identifying immunologic changes within the tumor microenvironment that correlates with those in the systemic circulation, which can then be used to monitor patients with metastatic disease. We found an increased frequency of CD4 T cells expressing high levels of the CD28/CTLA-4 homolog ICOS as well as a decreased frequency of FOXP3-expressing CD4 T cells within tumor tissues of treated patients. The $CD4^+$ICOShi population contained effector T cells that produced interferon (IFN)-γ and recognized the cancer-testis antigen NY-ESO-1 expressed on tumor cells. We therefore identified an increase in the ratio of ICOS-expressing effector to FOXP3-expressing regulatory T cells in tumor tissues of treated patients. Real-time polymerase chain reaction analyses also revealed changes in tumor tissues of treated patients consisting of increased T-bet and IFN-γ mRNA. These changes led to an increase in the ratio of the Th1 cytokine IFN-γ to the Th2 cytokine interleukin-10, which was consistent with an increase in the ratio of effector-to-regulatory T cells.

Immunologic changes within tumor tissues correlated with changes within peripheral blood in that an increased frequency of $CD4^+$ICOShi T cells was also detectable in the systemic circulation of treated patients. Therefore, we examined peripheral blood samples from metastatic melanoma patients who were treated with anti – CTLA-4 and found that sustained elevation of $CD4^+$ ICOShi T cells within the systemic circulation correlated with improved survival. These are the first results to demonstrate changes within the tumor microenvironment as a result of anti – CTLA-4 therapy that can be correlated with changes within the systemic circulation, which may potentially correlate with clinical benefit.

Radioprotection in Normal Tissue and Delayed Tumor Growth by Blockade of CD47 Signaling

*David R. Soto-Pantoja**, Justin B. Maxhimer*, Jeff S. Isenberg†, Lisa A. Ridnour‡, Hubert B. Shih*, William G. DeGraff‡, Maria Tsokos*, David A. Wink‡, David D. Roberts*. **Laboratory of Pathology, CCR;* ‡*Radiation Biology Branch, NCI, National Institutes of Health, Bethesda, MD;* †*Hemostasis and Vascular Biology Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA.*

Ionizing radiation (IR) is a major component of the therapeutic strategy for more than half of all cancer patients. Even though this approach is successful for many tumors, it is associated with harmful side effects resulting from damage to noncancerous tissue, which ultimately limits the effective radiation dose that can be delivered to a tumor. Here we describe a novel radioprotection strategy based on our previous finding that thrombospondin-1 and CD47 null mice are resistant to radiation injury. Mice bearing syngeneic B16 melanoma tumors in the hind limb were treated with CD47 antisense morpholino or vehicle followed by IR (10 Gy). Vehicle-treated mice showed increased numbers of apoptotic cells in surrounding tissues when compared with CD47 morpholino treatment. Remarkably, treatment with CD47 and IR caused a significant decrease in tumor regrowth when compared with IR only group. Similar results were observed in a syngeneic murine squamous cell carcinoma. Delay of tumor regrowth after IR by CD47 suppression is not due to increased radiosensitivity of the tumor cells because in vitro blockade of CD47 in B16 cells slightly increased their radio resistance. Another possible explanation is the enhancement of antitumor immunity. Immunostaining with the macrophage marker, CD68, revealed increased macrophage numbers in the periphery of tumors subjected to CD47 suppression, suggesting that treatment enhances recruitment and/or survival of macrophages, which could enhance clearance of irradiated tumor cells. Further evidence for a role of antitumor immunity came from the loss of the enhanced tumor radiation response after CD47 morpholino treatment when the squamous cell carcinoma was grown in athymic mice. Therefore, the efficacy of CD47 suppression in the syngeneic tumor models may be due in part to the enhancement of T-cell – mediated immunity. This suggests that CD47 morpholino treatment is radioprotective for T cells and may enhance cytotoxic T-cell activity post-IR. These results indicate that therapeutics targeting of CD47 may improve the response of the tumor microenvironment post-IR by enhancing innate and adaptive immune responses. On the basis of their supportive effects on peritumoral cells and tissues, CD47 antagonists may enable more aggressive application of IR to increase response rates for the large number of cancer patients that receive radiotherapy.

MONOCLONAL ANTIBODIES/ COMBINATIONS

Impaired Humoral Response to Influenza Vaccine and Prolonged B Memory Cell Depletion as a Consequence of Rituximab-based Immunochemotherapy in non-Hodgkin Lymphoma Patients

*Davide Bedognetti**†, Gabriele Zoppoli*†, Carlotta Massucco‡, Simonetta Zupo‡, Mario Roberto Sertoli*‡, Elisa Zanardi*, Marco Messina*, Guido Siffredi*, Andrea Bruzzone*, Enrico Balleari*, Manlio Ferrarini*‡, Alessio Nencioni*, Graziano Caltabiano*, Nicoletta Provinciali*, Omar Racchi*, Anna Maria Ferraris*‡, Pietro Blandini*, Francesco Boccardo*‡, Laura Sticchi*, Giancarlo Icardi*, Francesco Marincola†, Sabrina Bacilieri*, Filippo Ansaldi*, Andrea De Maria*‡. **University of Genoa;* ‡*National Cancer Research Institute of Genoa, Genoa, Italy;* †*National Institutes of Health, Bethesda, MD.*

Background: Rituximab, anti-CD20 monoclonal antibody, has become an essential drug for the treatment of non-Hodgkin

lymphoma (NHL). Although transient B cells depletion frequently occurs after rituximab treatment, it usually resolves after 6 to 9 months. Nevertheless, the high frequency of non-neutropenic infections and persistent hypogammaglobulinemia during the follow-up period, have been recently reported. The influenza vaccination is generally recommended in lymphoma patients, but no data are available about the activity of this vaccine after rituximab-based chemotherapy (RIT).

Objective: To assess the humoral response to the influenza vaccine after RIT in complete remission NHL patients, compared with healthy individuals.

Patients and Methods: Complete remission NHL patients who had completed RIT no less than 6 months before the accrual were eligible. Healthy volunteers served as an age-matched control group. The subjects were vaccinated with the same influenza vaccine (contained 2 A and 1 B viral strains). Hemagglutinin inhibition assays were performed before and 4 weeks after the vaccination. Seroconversion rate, seroprotection rate, and mean fold increase after Beyer correction, were evaluated to compare the 2 groups. Circulating lymphocytic subpopulations were assessed by immuno-cytofluorometry to evaluate the presence of potentially relevant phenotypic perturbations after vaccination and between the 2 groups.

Results: During the 2008/09 epidemic season, 31 patients and 34 healthy controls were enrolled and analyzed. The median period after RIT administration was 29 months (range, 7-54). Seroconversion rate, seroprotection rate, and mean fold increase after Beyer correction, were lower in the patient group compared with the healthy control group ($P < 0.05$ or less in 7/9 evaluated parameters) (Table 1). While peripheral $CD27^-$-naive B cells were present, $CD27^+$ memory B-cell populations were significantly depleted in the patients ($P < 0.0001$).

TABLE 1. (Bedognetti)

Antigen	Parameters	Patient Group	CTR	*P*
AH1N1	SC	29.0	41.2	NS
	SP	74.2	94.1	< 0.05
	BMFI	1.84 ± 0.92	4.69 ± 1.54	< 0.0001
AH3N2	SC	22.6	52.9	< 0.05
	SP	64.5	94.1	< 0.01
	BMFI	0.6 ± 0.9	1.9 ± 1.25	< 0.0001
B	SC	3.2	29.4	< 0.01
	SP	22.6	44.1	NS
	BMFI	0.59 ± 0.7	1.84 ± 0.91	< 0.0001

BMFI indicates mean fold increase after Beyer correction; CTR, control group; SC, seroconversion rate; SP, seroprotection rate.

Conclusions: Patients treated with RIT have a significant deficit in humoral response to the influenza vaccine compared with healthy controls, even a long time after treatment administration. In these patients, the vaccination does not appear to confer adequate protection. The profound depletion in $CD27^+$ B memory cells observed in these patients may explain this humoral failure.

FCγR Mediated Regulation of Adaptive Immunity: Implications for Antibody Therapies

Madhav Dhodapkar, Kavita Dhodapkar. *Yale University, New Haven, CT.*

Monoclonal antibodies (mAbs) have emerged as effective therapeutics that can mediate durable responses against cancer. Antitumor properties of mAbs can be mediated by several mechanisms including direct effects on tumor cells, and induction of innate immune mechanisms such as antibody-mediated cytotoxicity (ADCC). However, the potential capacity of mAbs to recruit adaptive immunity has received less attention. In earlier studies, we and other investigators have shown that uptake of tumor cells opsonized with antitumor mAbs by dendritic cells (DCs) leads to greatly enhanced cross presentation and induction of T-cell immunity. This process requires the engagement of Fc receptors on DCs. The capacity of DCs to induce immunity also depends on their maturation status, which in turn regulates the nature of induced immune responses. DCs are efficient not only at inducing $CD4^+$ T helper and $CD8^+$ killer T cells, but also FOXP3+ regulatory T cells, and IL17-producing inflammatory T cells. DCs express both activating and inhibitory Fcγ receptors and the balance of signaling via these receptors can regulate DC activation. Selective blockade of inhibitory FcγRIIB on human DCs leads to a distinct form of maturation, with the induction of type I interferon response genes and several inflammation-associated cytokines and chemokines. This induction of a type I interferon response is critical to FcγR-mediated DC maturation. FcγR-mediated activation also has a clear impact on the capacity of tumor-loaded DCs to induce immunity. In particular, such DCs lead to greater activation of IFNγ-producing effector T cells, with less concurrent activation of FOXP3+ Tregs. Recent studies of patients treated with antitumor mAbs support the concept that these agents may induce adaptive immunity. These preliminary studies also suggest that the analysis of immune responses in the tumor bed may be essential to better understand the effects of mAbs on adaptive immunity in vivo. Together, these data suggest that targeting tumor antigens selectively to activating FcγRs on human DCs can greatly enhance DC-mediated induction of antitumor T cell responses. This pathway may also be exploited to enhance the efficacy of antitumor monoclonal antibodies in cancer.

Enhancing Cancer Vaccines

Glenn Dranoff. Dana-Farber Cancer Institute, Boston, MA.

Efficacious cancer immunotherapies will likely require combinations of strategies that enhance tumor antigen presentation and antagonize negative immune regulatory circuits. We demonstrated that vaccination with irradiated, autologous melanoma cells engineered to secrete GM-CSF followed by antibody blockade of CTLA-4 accomplishes clinically significant tumor destruction with minimal toxicity in a majority of stage IV metastatic melanoma patients. The extent of tumor necrosis in post-treatment biopsies was linearly related to the natural logarithm of the ratio of infiltrating $CD8^+$ effector T cells to FoxP3+ Tregs, suggesting that further Treg inhibition might increase the frequency of clinical responses. Through an analysis of cytokine-deficient mice, we delineated a critical role for GM-CSF in Treg homeostasis. GM-CSF is required for the expression of the phosphatidylserine-binding protein MFG-E8 in antigen presenting cells, whereas the uptake of apoptotic cells by phagocyte-derived MFG-E8 maintains peripheral Treg numbers through TGF-β, MHC class II, and CCL22. In wild-type mice, MFG-E8 restrains the potency of GM-CSF-secreting B16 melanoma vaccines through Treg induction, whereas a dominant negative MFG-E8 mutant (RGE) potentiates therapeutic immunity through Treg inhibition. In patients, MFG-E8 is expressed at high levels in melanoma cells and/or tumor-associated myeloid elements upon progression to the vertical growth phase. MFG-E8 acts as a melanoma promoter through coordinated avb3 integrin signaling in the tumor microenvironment, thereby stimulating melanoma cell resistance to apoptosis, epithelial-to-mesenchymal transition, invasion, angiogenesis, and immune suppression. Inhibition of MFG-E8 with shRNAs or systemic antibodies enhances the antitumor activity of cytotoxic treatments in vitro and in vivo. Together, our results suggest that MFG-E8 blockade might prove therapeutic through both immune-mediated and tumor cell autonomous pathways.

Immune Activation by Cetuximab Involves NK Cells, CTL, and DC Against EGFR in Head and Neck Cancer Patients

Robert L. Ferris. Cancer Immunology Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA.

Immunotherapy with the EGFR-specific mAb cetuximab is clinically effective in 10% to 20% of patients with squamous cell carcinoma of the head and neck (SCCHN). Little information is available about the mechanism(s) underlying the patients' differential clinical response to cetuximab-based immunotherapy, although this information may contribute to optimizing the design of cetuximab-based immunotherapy. Our understanding of these mechanisms would benefit from the characterization of the variables which influence the extent of cell-dependent lysis of SCCHN cells incubated with cetuximab in vitro. The extent of NK cell lysis of SCCHN cells was influenced by the EGFR expression level, cetuximab concentration, and FcγR polymorphism. Effector cells expressing the FcγR IIIa-158 VV allele were significantly ($P < 0.0001$) more effective than those expressing FcγR IIIa VF and FF alleles in mediating lysis of SCCHN cells, expressed higher levels of the activation markers CD69 and CD107a, and secreted significantly ($P < 0.05$) larger amounts of inflammatory cytokines and chemokines. A murine SCCHN xenograft model was used to distinguish the antitumor contributions of cetuximab and FcγR IIIa-expressing NK cells. As other FcγR genotypes correlate with the clinical activity of cetuximab, we also show that cetuximab induces EGFR-specific CTLs in treated SCCHN patients, versus cetuximab-naive patients. Enhanced cross-presentation and DC activation were observed in the presence of NK cells and cetuximab. These results support a potential role for immune activation in SCCHN patients and may explain patient variability of cetuximab-mediated clinical responses. We will discuss the role of chemoradiotherapy combinations on cetuximab-mediated antitumor immunity, as their regimens are commonly used in the clinic. Cellular and secreted immune profiles and FcγR genotypes from the patients' lymphocytes may provide clinically useful biomarkers of immune activation in cetuximab-treated patients.

Can B Cells, the Small Ignored Guys Hidden in Melanoma Tissues, put us on the Right Track for Tumor-specific Antigens?

*Beatrix Kotlan**†, Mepur H. Ravindranath‡, Jozsef Timar§, Mark C. Glassy†. **Surgical and Molecular Tumorpathology, National Institute of Oncology; §2nd Department Pathology, Semmelweis University, Budapest, Hungary; †Integrated Medical Sciences Association, San Diego; ‡Terasaki Foundation Laboratory, Los Angeles, CA.*

Background and Objectives: Antibodies targeting specific biomarkers of proliferation and metastasis may render potential benefit for the treatment of cancer by stabilizing the disease and improving survival. The objective of this study is to isolate antibodies and construct antibody fragments from tumor-infiltrating B (TIL-B) cells capable of producing antibodies against such specific biomarkers.

Methods and Materials: Tumor tissues of superficial and nodular melanomas (n = 6) were dissected by regular surgery and processed for molecular genetic techniques. The TIL-B cells were defined by immunohistochemistry. Rearranged immunoglobulin heavy (VH-JH), κ (Vk-Jk), and λ (Vl-Jl) light chains were amplified by reverse transcription-polymerase chain reaction. Single-chain Fv (scFv) antibody fragment phages display libraries that were generated in suitable vector (PHEN1, pCANTAB) and bacterial (TG1 *Escherichia coli*) systems, enriched and selected for tumor binder capacity in enzyme-linked immunosorbent assay against native membrane fractions prepared from fresh cultivated cancer cells of different tissue origins. Selected tumor binder single-chain Fv antibody fragments were transformed into other bacterial systems to produce large-scale and purified antibody fragments, ready for further more exact specificity testing.

Results: The nature of the targeted tumor-associated antigens could be defined by enzyme-linked immunosorbent assay blocking, western blot, thin-layer chromatography, dot blot, and biochemical assays. A comparative DNA sequence analysis of the tumor binder antibody fragments strengthened the findings regarding the target antigens. Positive reactions were obtained by immunofluorescense assay/fluorescence-activated cell sorter analysis and confocal laser microscopy using fresh melanoma cell cultures and melanoma cryostat sections, respectively, compared with the negative control cell lines and normal tissue sections.

Conclusions: The immunglobulin variable region gene fragments obtained from TIL-B cells have highly potential tumor-specific binding capacity to key tumor-associated biomarkers of malignancy (glycolipids and glycoproteins) unrelated to normal progenitor cells. These results provide scope for simultaneous generation of multiple antibodies for targeting tumor heterogeneity, arresting tumor progression and metastasis, and providing strategies for cancer treatment.

Acknowledgments: The work was supported by the Hungarian OTKA T048933 and American-Hungarian Fulbright No120610 grants of B. Kotlan.

Antitumor Activity of Cytotoxic T-lymphocyte Antigen-4 Blockade Alone or Combined With Paclitaxel, Etoposide, or Gemcitabine in Murine Models

Gregg Masters, Liliana Barreto, Emel Girit, Maria Jure-Kunkel. *Oncology Drug Discovery, Bristol-Myers Squibb, Princeton, NJ.*

To determine whether the antitumor activity of an anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody (CTLA-4 mAb) is synergized or inhibited by the addition of chemotherapeutic agents, CTLA-4 mAb was evaluated alone and in combination with paclitaxel (Pac), etoposide (Eto), or gemcitabine (Gem) in murine tumor models. M109 lung carcinoma, SA1N fibrosarcoma, and CT26 colon carcinoma models were chosen on the basis of different sensitivities to the chemotherapeutic agents and CTLA-4 blockade. All compounds were tested at their optimal dose and schedule. When used in combination, CTLA-4 mAb was initiated 1 day after the first dose of chemotherapy. The percentage of tumor growth inhibition and the number of days to reach target tumor size were used to evaluate efficacy. Antitumor activity was scored as complete regression (nonpalpable tumor for ≥ 2 assessments) or partial regression (50% reduction in tumor volume for ≥ 2 assessments). Synergy was defined as antitumor activity significantly superior ($P < 0.05$) to the activity of monotherapy with each agent. In the M109 subcutaneous tumor model, which is insensitive to CTLA-4 blockade and modestly sensitive to Pac, Eto, and Gem, borderline synergy was evident with the combination of CTLA-4 mAb and Pac, whereas no effect was observed with Eto. Gem monotherapy did not produce significant M109 antitumor activity; however, combining Gem with CTLA-4 mAb resulted in synergy. In the M109 lung metastasis model, synergy was detected for CTLA-4 mAb combined with Eto, borderline synergy was found with Gem, and Pac did not enhance activity. SA1N fibrosarcoma is sensitive to CTLA-4 blockade and all 3 chemotherapies. Pac, Eto, and Gem enhanced the activity of CTLA-4 mAb in this model, but synergy was only observed with Eto. CTLA-4 mAb and Pac were ineffective against established CT26 colon carcinoma tumors, but synergistic when the tumor burden was minimal. Both Eto and Gem were effective as single agents in this model and the activity of both was significantly synergized by CTLA-4 mAb. In summary, the addition of CTLA-4 mAb to Eto, Gem, or Pac resulted in model-dependent synergistic activities. Synergy was observed regardless of the immunogenicity of the tumor and only when at least 1 of the therapies was active. All combination regimens were well tolerated and the chemotherapies did not appear to inhibit CTLA-4 mAb activity in the SA1N tumor model. What is of particular importance is that synergy was observed in tumors unresponsive to CTLA-4 mAb alone, suggesting that the chemotherapeutic agents might have induced immunogenic cell death. These findings provide support for the evaluation of chemoimmunotherapy combinations in clinical trials.

Development of Streptococcal Superantigen-derived Anticancer Immunotherapeutics

*Kelcey G. Patterson**, Jennifer L. Dixon*†, Peter S. Bastedo*, Shipa S. Gupta*, John K. McCormick*†. **Microbiology and Immunology, University of Western Ontario; †Lawson Health Research Institute, London, ON, Canada.*

Bacterial superantigens (SAgs), such as streptococcal pyrogenic exotoxin C (SpeC), are microbial toxins that unconventionally activate large numbers of T cells by cross-linking T-cell receptors with major histocompatibility complex class II molecules on antigen-presenting cells. In this study, a SAg-derived immunotherapeutic is being developed to localize and augment T-cell activation at the tumor site. Flow cytometry analysis assessed the cytotoxicity of mutated SpeC proteins on human colorectal cancer cells upon incubation with peripheral blood mononuclear cells. Mutations in SpeC that reduced binding affinity for major histocompatibility complex class II, or the T-cell receptors, subsequently decreased the activation and tumor cytotoxicity of peripheral blood mononuclear cells. In addition, JAM assay analysis demonstrated that the activity of modified SpeC proteins were still able to induce immune-mediated cancer cell death in vitro; however, they had a lower ability to elicit immune activation in comparison with SpeC wild type. To target SpeC at the cancer cells, a single-chain variable fragment (scFv) antibody was generated. The scFv was designed to recognize the tumor-associated antigen 5T4, a transmembrane glycoprotein frequently expressed on various carcinomas. Targeting of scFv5T4 has been verified with colorectal cancer cell lines known to express 5T4 by using a monomeric red fluorescent protein: scFv5T4 construct. Furthermore, HEK293 cells engineered to express 5T4:GFP provided additional support that scFv5T4 specifically targets its antigen. The scFv5T4 and modified SpeC were conjugated to form a fusion construct for the generation of a potential anticancer immunotherapeutic. To date, pilot data have shown that both the specificity of scFv5T4 and the cytotoxicity elicited by SpeC remain functional in vitro as a fusion protein. Future in vitro and in vivo studies will further assess the overall potential of the fusion construct and will aid in the development of novel SAg-based anticancer immunotherapeutics.

Synergy of Radiation and Immune Therapy in Tumor Eradication

*Maria Grazia Ruocco**, Noriko Kawashima†, Julie Huang*, Silvia Formenti‡, Michael L. Dustin*, Sandra Demaria†. **Molecular Pathogenesis; †Pathology; ‡Radiation Oncology, NYU School of Medicine, NY.*

We have investigated the mechanism of synergy between anti-(cytotoxic T lymphocyte antigen-4) CTLA-4 immunotherapy and radiation therapy (RT) in the treatment of the 4T1 mouse model of breast carcinoma. We have used genetically manipulated mice in which CXCR6 is replaced with green fluorescent protein and intravital 2-photon laser scanning microscopy with cyan fluorescent protein expressing tumors to follow the interaction of infiltrating $CD8^+$ T cells with tumor cells. We have found that the blocking anti – CTLA-4 antibody, 9H10, increased T-cell speed and effectively prevented stable interactions with tumor cells or stromal elements, critical for efficient killing. RT also increased T-cell velocity, but not to the same extent as the 9H10 treatment. When RT was combined with 9H10, we observed increased T-cell arrest in contact with tumor cells and later on elimination of the tumor. It has been shown that CTLA-4 ligation can increase T-cell motility and this can overcome a T-cell receptor-mediated stop signal. We confirmed that 9H10 treatment increased T-cell motility on ICAM-1-coated surfaces and 9H10 was dominant over stop signals provided by soluble anti-CD3 antibodies. However, when a 9H10 go signal was subordinated to a stronger stop signal provided by solid-phase anti-CD3, T cells did stop. On the basis of these observations, we suggest that anti – CTLA-4 therapy may impair killing within tumors by suppressing immunologic synapse formation, but this effect can be overcome by boosting tumor antigen presentation with RT.

Nanocell Delivery of PHA-665752 and Cetuximab Enables High-Efficacy Photodynamic Therapy Combination Treatment for Pancreatic Cancer in Preclinical Mice Model

Lei Z. Zheng, Prakash Rai, Conor Evans, Bryan Spring, Tayyaba Hasan. *Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA.*

Purpose: Photodynamic therapy (PDT) is an effective treatment for pancreatic cancers clinically. To optimize the PDT treatment for pancreatic cancer, we implemented an innovative drug delivery vehicle, referred to as "nanocell" and tested whether effective PDT combination treatment targeting MET and epidermal growth factor receptor (EGFR) pathways can be achieved.

Materials and Methods: We designed and synthesized a PHA nanocell containing the PDT photosensitizer, BPD, and a MET inhibitor, PHA-665752. A PHA nanocell is composed of a PLGA polymer nanoparticle core containing PHA-665752, and an outside lipid bilayer envelope containing BPD. The PHA nanocell was tested on the MET-positive pancreatic cell line, AsPC-1, and the orthotopic tumor for a combination treatment effect in vitro and in vivo, respectively.

Results: First, we found that the PHA nanocell can efficiently deliver the photosensitizer into cells. The nanocells deliver PHA-665752 into the cells and can effectively inhibit MET phosphorylation. Second, we compared the in vitro PDT/MET combination treatment efficacy of nanocell delivery with conventional drug delivery and found that the PHA nanocell offers synergistic PDT/MET combination treatment effect in killing cancer cells. Notably, when equal amounts of BPD and PHA-665752 are delivered into cells together but not coencapsulated in nanocells, little treatment benefit was observed when compared with BPD alone, therefore suggesting the synergy of the PDT/MET combination treatment owes to the unique drug delivery method. Third, we tested the PHA nanocell PDT/MET combination treatment in a MET-positive orthotopic pancreatic cancer model in vivo. Our results indicate that the PHA nanocell offers the most effective treatment efficacy in reducing the local tumor burden, when compared with BPD alone and BPD + nanoparticle, PHA-665752. Besides the superior treatment efficacy, the PHA nanocell also significantly reduced the systemic toxicity of PHA-665752, and allowed higher payload of PHA-665752 to be administered.

Conclusions and Future Direction: We have successfully implemented the nanocell drug delivery system into the PDT combination treatment of pancreatic cancer. We found that nanocells containing the MET inhibitor offered a significant improvement in PDT treatment efficacy compared with the conventional PDT/MET combination treatment both in vitro and in vivo. Currently, we are exploring the potential of incorporating cetuximab, a pharmaceutic inhibitor of EGFR, into the PHA nanocell so that the MET and EGFR crosstalk can both be targeted in combination with PDT treatment.

REGULATORY AND ACTIVATED T CELL SUBSETS

Identification of Heme Oxygenase-1-specific Regulatory $CD8^+$ T Cells in Cancer Patients

*Mads H. Andersen**, Rikke B. Sørensen*, Marie K. Brimnes*, Inge Marie Svane*†, Jürgen C. Becker‡, Per thor Straten*. **Center for Cancer Immune Therapy (CCIT); †Department of Oncology, Herlev University Hospital, Herlev, Denmark; ‡Department of Dermatology, University Hospital Würzburg, Würzburg, Germany.*

T regulatory cell (Treg) deficiencies are associated with autoimmunity. Conversely, $CD4^+$ and $CD8^+$ Tregs accumulate in the tumor microenvironment and are associated with the prevention of antitumor immunity and anticancer immunotherapy. Recently, $CD4^+$ Tregs have been much studied, but little is known about $CD8^+$ Tregs and the antigens they recognize. Here, we describe what we believe to be the first natural target for $CD8^+$ Tregs. Naturally occurring human leukocyte antigen-A2 – restricted $CD8^+$ T cells specific for the antiinflammatory molecule, heme oxygenase-1 (HO-1), were able to suppress cellular immune responses with outstanding efficacy. HO-1 – specific $CD8^+$ T cells were detected ex vivo and in situ among T cells from cancer patients. HO-1 – specific T cells isolated from the peripheral blood of cancer patients inhibited cytokine release, proliferation, and cytotoxicity of other immune cells. Notably, the inhibitory effect of

HO-1 – specific T cells was far more pronounced than that of conventional $CD4^+CD25^+CD127^-$ Tregs. The inhibitory activity of HO-1 – specific T cells seemed at least partly to be mediated by soluble factors. Our data link the cellular stress response to the regulation of adaptive immunity, expand the role of HO-1 in T-cell – mediated immunoregulation, and establish a role for peptide-specific $CD8^+$ T cells in regulating cellular immune responses. Identification of potent antigen-specific $CD8^+$ Tregs may open new avenues for therapeutic interventions in both autoimmune diseases and cancer (*J Clin Invest*. Epub ahead of print).

Cutaneous T Regulatory Cell Frequency Predicts the Magnitude of Tumor Vaccine-induced Memory Responses

Yushe Dang, Lupe G. Salazar, Doreen Higgins, Janice Morihara, Meredith Slota, Jennifer Childs, Nicole Bates, Emily Jackson, Mary L. Disis. *Tumor Vaccine Group, University of Washington, Seattle, WA.*

Cutaneous delayed-type hypersensitivity (DTH) reactions indicate the development of a tumor antigen-specific memory T-cell response after immunization. T regulatory cells (Tregs) play an important role in the regulation of immune responses and are found in abundance in the skin. We questioned whether the frequency of cutaneous Treg cells at a DTH site via antigen challenge after vaccination impacted the development of tumor vaccine – induced T-cell memory responses.

Methods: A DNA plasmid-based HER2 intracellular domain vaccine was given monthly for 3 months to patients with advanced stage HER2 overexpressing breast cancer in a phase I clinical trial. Six months after the last vaccine, a HER2 intracellular domain peptide mixture was injected intradermally to elicit a DTH response. DTH responses to the HER2 antigens were measured after 48 hours, and skin biopsies were then taken of the DTH site for immunohistochemistry analysis of FOXP3+ and $CD4^+$ T cells. The patients' blood was taken at the same time for measuring HER2-specific responses using an interferon-γ enzyme-linked immunosorbent spot assay.

Results: The numbers of Foxp3+ cells/slides were significantly decreased in DTH+ patients compared with normal skin controls (mean ± SE: DTH+, 4.5 ± 1; control, 18.5 ± 3.4; n = 5, $P = 0.004$), and DTH− patients (DTH− 13.2: ± 3.4, n = 5, $P = 0.047$). The greater the HER2 peptide-induced DTH responses, the lower the numbers of Treg+ cells at the DTH site ($P = 0.081$, $r = 0.567$). The numbers of FOXP3+ cells at the DTH site also reversely correlated with the magnitude of the HER2-specific T cells in peripheral blood mononuclear cells ($P = 0.085$, $r = 0.644$). In contrast, FOXP3+ cells at the DTH site did not correlate to a tetanus vaccine-induced response ($P = 0.644$, $r = 0.195$).

Conclusions: The frequency of Treg cells at a DTH site is inversely correlated with the magnitude of tumor vaccine-induced memory T-cell responses.

Evaluation of T Regulatory Cells in Melanoma Patients Treated With Dendritic Cell Vaccines and Interleukin-2 +/ − Metronomic Cyclophosphamide

Eva Ellebaek*†, *Lotte Engell-Nørregård*†, Jon Bjørn*, Marie Brimnes*, Kirsten Nikolajsen*, Mads Hald Andersen*, Per thor Straten*, Inge Marie Svane*†. *Department of Hematology, Center for Cancer Immune Therapy (CCIT); †Department of Oncology, University Hospital Herlev, Herlev, Denmark.*

Background: Regulatory T cells (Tregs) are a subtype of the $CD4^+$ lymphocytes that are characterized by $CD4^+CD25(high)CD127^-$/FoxP3+ expression. Their main function is to generate immune tolerance and protection against autoimmune diseases. Tregs are also involved in tolerance toward the cancer cells; they are found in increased numbers in cancer patients and are associated with poor prognosis in most cancers.

Ghiringhelli et al have shown that the amount of Tregs can be decreased by treatment with metronomic cyclophosphamide (MC). The Tregs are selectively targeted and their suppressive function is inhibited by this treatment. We have tested MC as additional therapy in connection with dendritic cell (DC) vaccination and low-dose interleukin (IL)-2.

Materials and Methods: Peripheral blood samples from melanoma patients treated with DCs were analyzed for absolute lymphocyte count, $CD4^+$, and $CD4^+CD25^+CD127^-$ cells by flow cytometry. The DC vaccine was administered in 2 different regimens and the results were compared.

Results: In the first trial, melanoma patients were treated with DC vaccines in combination with IL-2 2 MIU for 5 days after each vaccine and interferon-α2b 3 MIU at the time of each vaccine. The treatment was associated with a marked increase in the proportion of Tregs from baseline to the fourth vaccine. This was followed by a decrease in the proportion of Tregs to near baseline after the sixth vaccine.

In the second trial, melanoma patients were treated with DCs and IL-2 in the same dosage and schedule as described above but combined with MC 50 mg twice a day for 1 week altering with 1 week off treatment and a Cox2 inhibitor (Celebra) 200 mg daily. The same marked increase in Tregs from the first to the fourth vaccine was observed but surprisingly only a slight decrease in Tregs after the sixth vaccine was detected. Functional assays are ongoing to confirm the functionality of the Tregs.

No significant difference in the overall lymphocyte or $CD4^+$ T-cell count was found between the 2 regimens.

Conclusions: The MC-induced reduction in Tregs described by Ghiringhelli et al was not found in our setting. A possible explanation could be the concomitant use of IL-2 and we are now preparing a new DC vaccination trial in which MC but no IL-2 will be used.

CTREGS (Circulating Regulatory T Cells) Reduction in Melanoma Patients Treated With Intravenous High-dose Interferon-α2B

*Giusy Gentilcore**, Maria L. Ascierto*,†, Maria Napolitano*, Marilena Capone*, Ester Simeone*, Antonio Da Ponte*, Corrado Caraco'*, Ena Wang†, Nicola Mozzillo*, Francesco Marincola†, Paolo A. Ascierto*. **National Cancer Institute, Naples, Italy; †National Institutes of Health, Bethesda, MD.*

Introduction: T regulatory (Treg) cells are immunoregulatory cells involved in host's protection from autoimmune diseases by suppressing autoreactive T-cell responses. $CD4^+$ FoxP3+ Tregs are the best characterized Treg population among a large group of CD4 and CD8 cells with suppressive functions. Recent evidences show an overrexpression of Treg lymph nodes containing metastatic melanoma, (where they seem to inhibit the function of effector T cells infiltrating the tumor) and a subsequent Treg depletion leads to the restoration of antitumor immunity. In this study, we evaluate modifications in circulating Treg cells in 22 melanoma patients after the treatment with interferon-α2b, which is the only adjuvant therapy approved for use after surgery for high-risk cutaneous melanoma.

Methods: Analysis was performed on melanoma patients of National Cancer Institute of Naples. The patients were included in the study since July 2006 and they are a part of neoadjuvant or adjuvant treatment with intravenous HDI (20 MUI/m^2, 5 d/wk) for 4 weeks. Peripheral blood mononuclear cells were obtained from 22 consecutive melanoma patients. Blood draw was performed at days 0, 8, 15, 22, and 29. These cells were thawed and labeled with anti-CD4 and anti-CD25hi (BD, San Diego, CA) and anti-FoxP3 (eBioscience, San Diego, CA). Labeled cells were analyzed using a FACScalibur (Becton Dickinson).

Results and Discussion: Fifteen (68.2%) out of 22 patients showed a decrease of Treg cells in peripheral blood. The average value at day 0 for circulating Treg (cTreg) was 2.7%. The average percentage at day 29 was 1.4%. The average reduction was 1.4% (50% reduction

in the average value of cTreg). Statistical analysis showed an average decrease of 0.29% per week of treatment. Despite this clear trend in reducing cTreg by HDI, statistical significance was not reached (probably due to the power of the study). Moreover, great differences between the disease status, the prognosis (recurred/not recurred patients, alive/deceased), and an increased basal percentage of cTreg have been observed. These preliminary data show reduction in cTregs after the treatment with HID, although no conclusion about the role of such reduction in terms of response to treatment or as prognostic markers of better/worse disease can be inferred.

Human Peripheral Blood and Bone Marrow Epstein Barr Virus-specific T-cell Repertoire in Latent Infection Reveals Distinct Memory Subsets Based on Cytokine Profiles

*Manuel Guerreiro**, Il-Kang Na*†, Anne Letsch†, Doreen Haase*, Christian Meisel*, Sandra Bauer*, Petra Reinke‡§, Hans-Dieter Volk*§, Carmen Scheibenbogen*. **Institute of Medical Immunology;* †*Departments of Hematology and Oncology;* ‡*Nephrology and Internal Intensive Care;* §*Berlin-Brandenburg Center for Regenerative Therapy (BCRT), Charité Universitätsmedizin Berlin, Berlin, Germany.*

Epstein-Barr virus (EBV) infection leads to life-long viral persistence. Although EBV infection can result in chronic disease and malignant transformation, most carriers remain disease-free due to an effective control of the virus by T cells. EBV-specific interferon-γ (IFN-γ)-producing T cells could be demonstrated in acute and chronic infection by many researchers. Recent studies provide, however, evidence that assessing IFN-γ alone is insufficient to assess the quantity and quality of a T-cell response. In this study, we therefore analyzed EBV-specific T-cell responses in peripheral blood and bone marrow (BM) of healthy virus carriers by using multicolor flow cytometry and overlapping peptide pools of latent EBNA-1 and lytic BZLF-1 protein.

The majority of ex vivo EBNA-1 – reactive $CD8^+$ T cells as well as Epstein-Barr Virus Nuclear Antigen and BZLF-reactive $CD4^+$ T cells were interleukin 2 (IL-2) and tumor necrosis factor (TNF)-producing memory cells, the later being significantly more frequent in BM. After in vitro expansion, 7 different subsets could be distinguished based on their cytokine profile. One subset of EBV-specific $CD4^+$ and $CD8^+$ T cells displayed a differentiated effector IFN-γ+/TNF+ phenotype. However, a substantial number of EBV-specific $CD4^+$ and $CD8^+$ T cells retained a TNF single or TNF+/IL-2+ phenotype resembling early differentiated memory T cells. Interestingly, a large subset of EBNA-specific $CD4^+$ T cells in BM had a triple cytokine producer phenotype characterizing multifunctional memory T cells. Remarkably, both $CD4^+$ and $CD8^+$ T cells generated from BM revealed significantly higher cytotoxic potential. Sorting for the differentiation subsets revealed that EBV-specific T cells were predominantly expandable from the central memory compartment. Our data shows that triple-cytokine assessment of EBV-specific T cells is necessary to delineate the various subsets of EBV-specific memory T cells, which reflect the profile of a protective immune response.

Cytotoxic T Cells Strike Back: Activated $CD8^+$ T Effector/Memory Cells Eliminate $CD4^+$ $CD25^+$ FOXP3+ T Suppressor Cells From Tumors via FASL-mediated Apoptosis

Mehmet O. Kilinc, Rachael B. Turner, Tao Gu, Lauren P. Virtuoso, Nejat K. Egilmez. *Microbiology and Immunology, University at Buffalo, Buffalo, NY.*

Sustained intratumoral delivery of interleukin (IL)-12 promotes the activation of tumor-resident $CD8^+$ T effector/memory cells and induces apoptotic death of $CD4^+$ $CD25^+$ Foxp3+ T suppressor cells. Depletion of $CD8^+$ T cells before the treatment resulted in the abrogation of posttreatment T suppressor cell apoptosis revealing a link between $CD8^+$ T-cell activation and T suppressor cell elimination. IL-12 failed to induce T suppressor cell depletion in interferon (IFN)-γ or Fas ligand (FasL) knockout mice but was effective in perforin-knockout mice demonstrating a requirement for IFN-γ and FasL in T suppressor cell death. Adoptive transfer of wild-type $CD8^+$ T cells to FasL knockout mice restored treatment-induced T suppressor cell loss in tumors establishing that expression of FasL on $CD8^+$ T cells was sufficient to promote T suppressor cell apoptosis. IL-12 failed to up-regulate FasL on T effector cells in IFN-γ knockout mice demonstrating a requirement for IFN-γ in FasL up-regulation. Adoptive transfer of wild-type $CD8^+$ T effector cells to IFN-γ knockout mice resulted in the restoration of therapy-induced T suppressor cell death confirming that autocrine IFN-γ was sufficient to up-regulate FasL on $CD8^+$ T cells. These findings reveal a mechanism by which cytotoxic T cells can overcome preexisting regulatory mechanisms.

Human FoxP3+ T Regulatory Cells: Facts or Technical Artifacts?

*Maciej Kmieciak**, Madhu Gowda*, Laura Graham*, Kamar Godder*, Harry D. Bear*, Francesco Marincola†, Masoud H. Manjili*. **Virginia Commonwealth University Massey Cancer Center, Richmond, VA;* †*National Institutes of Health, Bethesda, MD.*

Background: FoxP3 has been suggested to be a standard marker for murine T regulatory cells whereas its role as marker for human T regulatory cells is controversial. Although some reports have shown that human FoxP3+ T cells had no regulatory function, others have shown their role in the inhibition of T-cell proliferation.

Methods: T-cell receptor activation was performed by means of brayostatin-1/Ionamycin as well as mixed lymphocyte reaction. Flow cytometry was performed to determine Foxp3 expression, cell proliferation, viabilities, and phenotype analyses of T cells.

Results: Both $CD4^+$ and $CD8^+$ T cells expressed FoxP3 upon activation in vitro. The expression of FoxP3 remained stable in $CD4^+CD25^+$ T cells whereas its expression in $CD8^+CD25^+$ T cells seemed to be transient. $CD4^+CD25^+$FoxP3+ T cells expressed CD44 and CD62L, showing their effector and memory phenotypes. The presence of $CD4^+CD25^+$FoxP3+ T cells at a biologically relevant ratio did not inhibit T-cell activation or proliferation. Viability of T cells was reduced upon activation and FoxP3 expression, perhaps due to activation-induced cell death.

Conclusions: The expression of FoxP3 does not convey a regulatory function in $CD4^+CD25^+$ T cells. The regulatory function of $CD4^+CD25^+$FoxP3+ T cells reported in some papers may be due to increased activation-induced cell death that could have bystander inhibitory effects, particularly when used at an artificial ratio that is far beyond biologically relevant ratios.

Helpless $CD8^+$ T Cells are not Hopeless

Maciej Kmieciak, Andrea Worschech†‡§, Madhu Gowda*, Amy Depcrynski*, Ena Wang†, Kamar Godder*, Shawn Holt*, Keith L. Knutson‖, Francesco Marincola†, Masoud H. Manjili*. *Virginia Commonwealth University Massey Cancer Center, Richmond, VA;* †*National Institutes of Health, Bethesda, MD;* §*Genelux Corp, San Diego, CA;* ‖*Mayo Clinic College of Medicine, Rochester, MN;* ‡*University of Wuerzburg, Wuerzburg, Germany.*

There are 2 models describing the role of $CD4^+$ T-cell help for the induction of effective $CD8^+$ memory T-cell responses. The programming model suggests that $CD4^+$ T cells are required during primary response to induce differentiation of memory T cells. The maintenance model suggests that $CD4^+$ T cells are required during recall response to support persistence and effector function of $CD8^+$ memory T cells, otherwise helpless $CD8^+$ T cells will undergo apoptosis mediated by the tumor necrosis factor-related apoptosis-inducing ligand. Using a mouse model of breast carcinoma, we showed that neu-specific helpless $CD8^+$ T cells can differentiate into memory T cells and maintain their effector function against

neu-positive primary tumors and against recall tumor challenge in the absence of $CD4^+$ T cells. $CD4^+$ helper T cells were only involved in phenotype differentiation of $CD8^+$ T cells and in preventing hyperactivation of $CD8^+$ T cells. We showed that activation of the interferon (IFN)γ-IFNγ Rα axis masks the antitumor efficacy of helpless T cells because of higher production of IFNγ by helpless T cells that facilitate neu antigen loss and tumor escape. Therefore, apparent differences in the antitumor ability of helped and helpless T cells during primary tumor challenge were not a result of the shortcomings of helpless T cells. These results show a previously unrecognized function of helpless $CD8^+$ T cells during primary and memory responses against breast cancer.

Antitumor Immunity Induced by Toll-like Receptor-7 Ligation is Limited by Inflammation-induced Immunosuppression but can be Restored through Interleukin-10 Blockade

Hailing Lu, Ekram Gad, Yi Yang, Amy Chang, Mary L. Disis. *Tumor Vaccine Group, University of Washington, Seattle, WA.*

Toll-like receptor (TLR) agonists play an important role in linking innate and adaptive immunity. Multiple TLR agonists have been shown to have an antitumor effect in animal models. However, the therapeutic efficacy of TLR agonist monotherapy in cancer patients has been limited. Studies from our group demonstrate that topical treatment with a TLR-7 agonist, imiquimod, can induce significant regression of breast cancer in neu-transgenic mice, a model of human HER2+ breast cancer. Topical imiquimod or control vehicle cream was applied to neu-transgenic mice at the onset of palpable spontaneous tumors. One treatment cycle includes 3 days of treatment followed by 4 days of rest. The treatment was repeated for up to 6 weeks. Imiquimod inhibited tumor growth. Gene expression analysis using tumor RNA showed that imiquimod activated multiple immune response pathways, resulting in a highly inflammatory microenvironment. Selective depletion experiments demonstrated that the antitumor effect was dependent on CD8 T cells, but not on CD4 or natural killer cells. Although tumor regression occurred, the cancers relapsed within a month of cessation of therapy. Further analysis revealed that imiquimod treatment induced both interleukin (IL)-10 gene expression in the tumor and elevated levels of circulating IL-10, in addition to T_H1 cytokines. $CD4^+CD25^+Foxp3+$ T regulatory cells increased in the tumor, and there was peripheral circulation after imiquimod treatment. Furthermore, there was a significant increase in CD4 T cells that were dual expressers of interferon-γ and IL-10. When mice were treated with an anti − IL-10 antibody, but not an anti-CD25 antibody, in combination with imiquimod, the antitumor effect was significantly enhanced compared with imiquimod alone. These data suggest that the excessive inflammation induced by TLR agonists may result in immunosuppression through IL-10 induction, and that blocking IL-10 may enhance the therapeutic efficacy of TLR agonists in treating established cancer.

Tumor-derived Adenosine Enhances Generation of Adaptive Regulatory T Cells (Tr1)

*Magis Mandapathil**†, Stephan Lang†, Edwin K. Jackson‡, Elieser Gorelik*, Theresa L. Whiteside*. **Pathology, UPCI; ‡Pharmacology, University of Pittsburgh, Pittsburgh, PA; †Otorhinolaryngology, University of Duisburg-Essen, Essen, Germany.*

Objective: $CD4^+CD25^-IL\text{-}10+$ adaptive regulatory T cells (Tr1) are generated in the peripheral blood upon encountering cognate antigens (Ag). In cancer patients, their frequency is increased; however, the mechanisms used by these cells to mediate suppression are not yet defined. The ectonucleotidases, CD39 and CD73, convert ATP into immunosuppressive adenosine, which binds to the A2a receptors on effector T cells, inhibiting their functions. These ectonucleotidases are known to be expressed in human natural T regulatory and tumor cells. In this study, we evaluated the effects of tumor-derived adenosine on the generation of Tr1 cells and the involvement of the adenosinergic pathway in Tr1-mediated immunosuppression.

Methods: To generate Tr1 cells, we used an in vitro model in which sorted $CD4^+CD25^-$ T cells were incubated with autologous immature dendritic cells and irradiated $CD73^+$ MDA tumor cells or $CD73^-$ MCF-7 tumor cells for 10 days in the presence of low doses of IL-2, IL-10, and IL-15 (10-15I U/mL). Expression of conventional Tr1 markers and ectonucleotidases on Tr1 cells was evaluated by multiparameter flow cytometry. Suppression mediated by the generated Tr1 cells was assessed in CFSE-based assays with OKT3/anti-CD28Ab-stimulated autologous $CD4^+CD25^-$ responder cells (RC). ATP hydrolysis was measured using a luciferase-based ATP detection assay. Adenosine present in cell supernatants was analyzed by mass spectrometry.

Results: Quantitatively, more Tr1 cells were generated in the $CD73^+$ ($Tr1TUCD73^+$) microenvironment than in the $CD73^-$ microenvironment ($Tr1TUCD73^-$) ($P < 0.01$). The $Tr1TUCD73^+$ showed increased suppression of autologous RC compared with $Tr1TUCD73^-$. $Tr1TUCD73^+$ hydrolyzed exogenous ATP at a higher rate ($P < 0.05$) and produced higher amounts of adenosine ($P < 0.05$) than $Tr1TUCD73^-$. ARL67156, an ectonucleotidase inhibitor, decreased the suppression of proliferating responder cells mediated by Tr1 (44% ± 2% vs. 17% ± 1%; $P < 0.01$). ZM241385, the A2A receptor antagonist, reduced the Tr1-mediated suppression from 44% ± 2% to 15% ± 3% ($P < 0.02$).

Conclusions: Tumor-derived adenosine favors the generation of immunosuppressive Tr1 cells. These Tr1 cells have increased CD39 and CD73 expression and elevated enzymatic activity compared with $Tr1TUCD73^-$ and control cells. The data suggest that adenosine plays a major role in the induction of Tr1 cells, which also produce and use adenosine to mediate suppression in the tumor microenvironment.

CD81—A New Functional Marker for Tumor-induced Regulatory T Cells That Suppress Priming of Tumor-specific Effector T Cells in Reconstituted, Lymphopenic Hosts

*Christian H. Poehlein**, Michael E. Affentoulis*, Daniel Haley†, Edwin B. Walker†, Bernard A. Fox*‡. **Laboratory of Molecular & Tumor Immunology; †Laboratory for Immunological Monitoring, Earle A. Chiles Research Institute; ‡Department of Molecular Microbiology and Immunology, Oregon Health & Science University (OHSU), OHSU Cancer Center, Portland, OR.*

We reported earlier that B16BL6-D5 (D5) systemic tumor-bearing mice (TBM) harbor $CD4^+CD25^+$ tumor-induced regulatory T cells that block vaccination-induced priming of tumor-specific T cells. Elimination of these cells restores priming and therapeutic efficacy in the reconstituted, lymphopenic mouse (RLM) model. Gene microarray analysis of purified TBM $CD4^+CD25^+$ T cells identified a number of selectively overexpressed genes, one of which was CD81. CD81 surface expression highly correlates with CD25 expression on CD4 T cells and is selectively up-regulated on TBM $CD4^+CD25^+$ T cells. Magnetic depletion of $CD81^+$ TBM T cells before reconstitution successfully restored the priming of therapeutic, tumor-specific effector T cells in the RLM model compared with naive donor T cells. Here we show that in vivo treatment with anti-CD81 monoclonal antibody in TBM donor mice before reconstitution of the lymphopenic host reduces the suppressive capacity of tumor-induced regulatory T cells. Interestingly, inhibition of CD81 in RLM hosts reconstituted with unmanipulated TBM T cells does not reduce the suppressive capacity of tumor-induced regulatory T cells. Thus, depletion or in vivo inhibition of CD81 on donor T cells before reconstitution reduces tumor-induced regulatory T cells and restores the priming efficacy of a successful tumor vaccine for donor T cells used in reconstitution. CD81 therefore serves as novel marker to manipulate donor T cells in the RLM model. Clinical trials using vaccination of lymphopenic cancer patients reconstituted with CD25-depleted peripheral blood mononuclear cells will allow further evaluation of CD81 as an interventional marker for tumor-induced regulatory T cells during the course of treatment.

Effective markers for tumor-induced regulatory T cells will be crucial for the development of future interventional immunotherapy protocols to enhance the generation of therapeutic effector T cells by tumor vaccines.

Supported by K22CA127739, RO1CA80964, the Murdock Trust and the Chiles Foundation.

Characterization of Immune Response During Osteolytic Progression of Breast Cancer

Anandi Sawant, D. Chanda, J. Hensel, S. Ponnazhagan. *Department of Pathology, University of Alabama at Birmingham, Birmingham, AL.*

Osteolytic bone metastasis is common in several human cancers, including carcinomas of the breast, lung, thyroid, and kidney. Dissemination of the primary tumor to the bone triggers the production of osteotropic cytokines and growth factors that not only result in osteoclast activation, but also promote the growth of tumor cells and immune suppression in the bone microenvironment. Conversely, products of bone cells are critical for normal development of the hematopoietic and immune systems, and provide a niche for long-term memory B and T cells. Thus, understanding the influence and interaction of metastasizing cancer cells with cells of the bone skeletal system and on cells of the immune system will provide clues toward designing preventive and therapeutic strategies for osteolytic bone metastasis. Female BALB/c mice were injected with 106 4T1 cells that constitutively express firefly luciferase via intracardiac route. Cohorts of mice were killed at the onset of organ metastasis and then as the tumor burden increased. Immunophenotyping was performed on cells obtained from the bone marrow and spleen to enumerate the number and maturation status of macrophages, myeloid dendritic cells, plasmacytoid dendritic cells (pDC), CD4, CD8, and natural killer cells by flow cytometry. MicroCT and histologic analysis were performed on femur and tibia. Results indicated that during the onset of metastasis there was a significant increase in pDC number and activation in both bone and spleen. The number of myeloid DC was increased only in bone, whereas CD4 population was increased only in spleen. However, as the tumor burden increased in the bone, the number of pDC decreased in bone, but their numbers remained high in spleen. There was no change in the numbers of CD4 cells in bone and their numbers remained elevated in spleen. CD8 cells were decreased in both spleen and bone during the early and late stages of bone metastasis. MicroCT analysis of bone showed significant destruction of tibia at the onset of metastasis and the skeletal damage was progressive in both femur and tibia as the tumor burden increased. TRAP staining revealed the presence of significantly high numbers of osteoclasts in areas surrounding the tumor. Taken together, these results suggest that in the initial phase of tumor dissemination, pDC skew the T-cell response more toward Th2 type, which facilitates tumor growth and metastasis. The activated T cells may produce cytokines such as interleukin (IL)-3, IL-17, and IL-11, which induce osteoclastogenesis and allow metastatic phenotype. Results of these ongoing studies will be further discussed.

Acute Inflammation With Interleukin-18/Interleukin-12 or α-GalCer Treatment Induces Liver iNKT Cell Apoptosis and Repopulation From Peripheral Tissues Whereas Chronic Inflammation Ablates Systemic iNKT Cells With Thymic-dependent Repopulation

Jeffrey J. Subleski, Veronica L. Hall, Anthony J. Scarzello, Thomas B. Wolfe, John R. Ortaldo, Deborah L. Hodge, Jonathan M. Weiss, Tim Chan, Robert H. Wiltrout. *NCI-CCR-LEI, Frederick, MD.*

We showed earlier that interleukin (IL)-18/IL-12 treatment of tumor-bearing mice elicited significant antitumor activity concurrent with a decrease in the detectable number of invariant natural killer (NK) T (iNKT) cells. We now show that acute treatment with IL-18/IL-12 initially decreased the number of NK1.1(+) iNKT cells whereas α-galactosylceramide (α-GalCer) decreased both NK1.1(+) and NK1.1(−) iNKT cells in the liver. Genomic quantitative polymerase chain reaction analysis of iNKT cells' restricted Vα14Jα18 T-cell receptor confirmed the loss of cells at 24 hours in the liver rather than receptor down-modulation. A small decrease or no change in the genomic levels of Vα14Jα18 from other lymphoid tissues indicates the loss of iNKT cells from IL-18/IL-12 or α-GalCer treatment of mice regulated by the liver microenvironment. Furthermore, an increase in Annexin V was detected on liver iNKT cells after treatment with IL-18/IL-12, and an increase in cells that were TUNEL positive was observed in the liver after α-GalCer treatment of mice. Although iNKT cells initially (24 h) decrease in the liver after acute administration of α-GalCer or IL-18/IL-12, these cells subsequently (72 h) reappear and expand in the liver. To study the expansion of iNKT cells in the liver, we administered bromodeoxyuridine to mice 24 hours after acute treatment with IL-18/IL-12 or α-GalCer and found that a high percentage of proliferating liver iNKT cells were recent emigrating NK1.1(−) iNKT cells that could account for the expansion in the liver.

Tumor-derived Microvesicles Induce, Expand, and Modulate Biologic Activity of Human $CD4^+CD25^+FOXP3+$ T Regulatory Cells

Marta Szajnik, Malgorzata Czystowska, Miroslaw J. Szczepanski, Magis Mandapathil, Theresa L. Whiteside. *University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

Background: Patients with cancer have higher $CD4^+$CD25 high T regulatory cell (Treg) frequency in peripheral blood than normal controls, and these Tregs mediate higher suppression. Body fluids of cancer patients also contain tumor-derived microvesicles (TMVs). Both Tregs and TMVs may be responsible for tumor escape. In this study, we tested the hypothesis that TMVs influence biologic activity of human $CD4^+CD25^+FOXP3+$ Tregs.

Methods: TMVs were isolated and purified from supernatants of the OVCAR3, SKOV3, AD10 (ovarian cancers), and PCI-13 (oral cavity cancer). The TMV molecular profile was checked by Western blot and by fluorescent-activated cell sorting. $CD4^+$CD25 high and $CD4^+CD25^-$ cells sorted by flow cytometry or expanded with rapamycin (1 nM) were cultured with TMV (5-60 μg/mL) for different time periods. Proliferation of freshly isolated or rapamycin-expanded Treg ± TMV was determined by cell counts and in CFSE assays. Apoptosis of cultured cells ± TMV was assessed by Annexin-V (ANX) binding. TMV-sensitive $CD8^+$ Jurkat cells were used as positive controls for TMV-mediated apoptosis. Treg suppressor function was tested in cocultures with CFSE-labeled OKT3-activated autologous $CD4^+CD25^-$ responder cells. Multicolor flow cytometry was used to phenotype Treg ± TMV. Culture supernatants were tested for levels of cytokines in Luminex-based assays.

Results: The TMVs were positive for expression of LAMP-1, MAGE3/6, transforming growth factor (TGF)-β1, major histocompatibility complex classes I and II, Fas ligand, and interleukin (IL)-10. TMV significantly enhanced the expansion of $CD4^+CD25^+FOXP3+$ T cells. In contrast, TMV impaired the proliferation of $CD4^+CD25^-$ T cells. TMVs also induced the conversion of $CD4^+CD25^-$ T cells into Tregs. TMV-activated Tregs showed an increased expression of Fas ligand, IL-10, TGF-β1, cytotoxic T lymphocyte anigen-4, granzyme B, and perforin relative to controls ($P < 0.05$). Purified Tregs were resistant to TMV-mediated apoptosis relative to Jurkat cells, which were sensitive (5% vs. 65% ANX binding cells, respectively). Tregs cocultured with TMVs mediated stronger suppression ($P < 0.01$) of responder cell proliferation. TMVs also increased the expression of phosphorylated SMAD2/3 and STAT3 in Tregs ($P < 0.05$). We further showed that neutralization of TGF-β1 and IL-10 significantly abolished TMV-mediated induction of Tregs.

Conclusions: Our data indicate that TMVs have immune-regulating properties. They promote Treg expansion, up-regulate suppressor function, and enhance the resistance to apoptosis of Tregs. The TMV–Treg interaction represents a newly defined mechanism that might be involved in the regulation of peripheral tolerance by tumors and immune evasion in cancer.

Regulation of Tumor Immunity by Natural Killer T-cell Subsets

Masaki Terabe. Vaccine Branch, National Cancer Institute, Bethesda, MD.

Natural killer T (NKT) cells and CD1d-restricted T cells have been shown to play critical roles in the regulation of tumor immunity. We have shown in several mouse tumor models that type I NKT cells expressing a semi-invariant T-cell receptor with Vα14Jα18 enhance tumor immunity, whereas type II NKT cells suppress tumor immunity when stimulated with agonistic antigen. Furthermore, the 2 types of NKT cells counteract each other, forming a new immunoregulatory axis. We have reported that type II NKT cells can suppress $CD8^+$ cytotoxic T lymphocytes by producing interleukin-13, which then induces production of transforming growth factor-β by $CD11b^+Gr-1+$ cells. We now observed that type II NKT cells also suppress $CD4^+$ T cells, suggesting that this axis impacts a broader range of immune responses. Alpha-galactosylceramide (α-GalCer) is a prototypic glycolipid antigen recognized by type I NKT cells. It has been reported that activation of type I NKT cells by α-GalCer and its analogs, such as OCH , can induce strong antitumor immunity despite the fact that they preferentially induce different cytokine profiles in activated type I NKT cells. Although many glycolipids have been tested for their ability to induce antitumor responses by activating type I NKT cells, those that induce strong antitumor immunity have α-linked galactose as a sugar moiety. We tested glycosylceramides with the same lipid structure having different sugar moieties and discovered that β-mannosylceramide (β-ManCer) induces anti-tumor immunity to a similar degree as α-GalCer in a type I NKT cell-dependent manner. However, they induced protection through different mechanisms; α-GalCer – induced protection was completed abrogated in interferon-γ KO mice, whereas β-ManCer – mediated protection partially remained in interferon-γ KO mice. This is the first evidence to our knowledge that a glycosylceramide with a β-linked sugar moiety can induce significant antitumor immunity, and the first description of a type I NKT cell agonistic antigen that induces protection by a mechanism(s) different from that induced by α-GalCer. We are currently investigating possible synergy between these 2 type I NKT cell agonists to induce protection, with the goal of developing more effective cancer immunotherapies.

Interleukin-1 Role in Human Th17 Responses, Dendritic Cell Activation, and Epithelial Cell Transformation

*Giorgio Trinchieri**, Marco Cardone*, Rosalba Salcedo*, Yava Jones*, Lyudmila Lyakh*, Christophe Cataisson†, Stuart Yuspa†. **Cancer and Inflammation Program, National Cancer Institute, Frederick; †Laboratory of Cell Biology and Genetics, National Cancer Institute, Bethesda, MD.*

We have shown that interleukin (IL)-1β production is essential for the induction of Th17 cell differentiation by human dendritic cells (DC) activated by the Dectin1 ligand β-glucan. We have now determined that a positive feedback through autocrine IL-1β and activation of nuclear factor-κB is necessary for transcription and production of late response cytokines by β-glucan – activated human DCs, including the Th17 amplifying cytokine IL-23. Thus, DC-produced IL-1β favors the Th17 response by acting both on the antigen-presenting cells and on the responding $CD4^+$ T cells. We have identified a similar autocrine-positive feedback of IL-1 with activation of nuclear factor-κB in epithelial cell transformation. The adaptor MyD88 is required for signaling through all toll-like receptors except toll-like receptor-3 and through the IL-1 receptor family. Studies by us and others have shown that expression of MyD88 is required for carcinogenesis in the skin, colon, and liver. In skin carcinogenesis, expression of MyD88 seems to be required for optimal papilloma formation in both radiosensitive hematopoietic cells and radioresistant host cells. To investigate whether signaling through MyD88 in keratinocytes was important for tumorigenesis, we transformed in vitro keratinocytes with oncogeneic ras and found that MyD88 expression in these cells was necessary for the production of chemokines, metalloproteases, and hematopoietic growth factors, and also for optimal growth when grafted in vivo. It was found that the lack of expression of MyD88 prevented the expression of the full transformation by blocking the ability of autocrine IL-1α to signal through the IL-1 receptor.

Interleukin-17-secreting CD8 T Cells' Functional Plasticity and Potential Role in Tumor Immunity

Charles G. Drake*†‡, *Hung-Rong Yen*§, Satoshi Wada*, Joseph Grosso*, Derese Getnet*, Monica Goldberg*, Kristin Pyle*, Drew Pardoll*, Ching-Tai Huang*‖. **Oncology, Johns Hopkins Kimmel Cancer Center; †Urology, Johns Hopkins-Brady Urological Institute; ‡Immunology, Johns Hopkins University, Baltimore, MD; §Pediatrics, Chang Gung Children's Hospital and College of Medicine; ‖Internal Medicine, Chang Gung Memorial Hospital, Taoyuan, Taiwan.*

Interleukin (IL)-17 – secreting CD8 T cells have been described in several settings, but little is known about their functional characteristics. Whereas Tc1 cells (CD8 T cells generated under conditions similar to classic CD4 Th1 cells) produce interferon (IFN)-γ and efficiently kill targets, Tc17 cells (CD8 T cells generated under conditions similar to CD4 Th17 cells) predominantly secrete IL-17 and lack lytic function in vitro. Interestingly, the small numbers of IFN-γ – secreting cells generated under Tc17 conditions also lack lytic activity, and express a pattern of cell surface proteins nearly identical to IL-17 – producing CD8 T cells. As is the case for IL-17 – secreting CD4 T cells (Th17), STAT3 is important for Tc17 polarization, both in vitro and in vivo. Adoptive transfer of pure populations of antigen-specific IL-17 – secreting Tc17 cells into antigen-bearing hosts resulted in near complete conversion to an IFN-γ – secreting phenotype, showing functional plasticity in this CD8 T-cell subset. In addition, Tc17 accumulated to a greater extent than Tc1 cells' adoptive transfer of CD8 T cells cultured under Tc17 conditions mitigates tumor outgrowth in vivo. Taken together, these data show that CD8 T cells, like CD4 T cells, are functionally plastic and that CD8 T cells cultured in the presence of transforming growth factor-β and IL-6 may play a role in tumor immunotherapy.

STEM CELLS/CIRCULATING TUMOR CELLS

Vinorelbine-induced Angiogenesis and Metastatic Spread in Nonsmall Cell Lung Cancer and Breast Cancer are Circumvented With Induced Pluripotent Stem Cells Encoded With Anti-GRP78 Small Hairpin RNA, Which Induces Apoptosis After a Gene-Silencing Bystander Effect

John Giannios, Emmanuel Michailakis, Nick Alexandropulos. *Oncology, GSHA, Athens, Greece.*

Introduction: Vinorelbine-tartrate in a small number of nonsmall cell lung cancer (NSCLC) and breast Cancer (Ca) cells induces tumor relapse with enhanced angiogenesis and metastasis by inducing an innate cancer cellular stress response, which enhances the expression of a prosurvival protein GRP78 that blocks cell death or apoptosis, increasing the growth and spread of NSCLC and breast Ca owing to chemoresistance. We aim to circumvent this with the use of induced pluripotent stem cells (iPSCs) encoded with antisense GRP78 small hairpin RNA (shRNA).

Materials and Methods: We took iPSCs, which we infected with a DNA vector that encoded an RNA molecule of 67 nucleotides. The sequence of this shRNA is designed to suppress the GRP78 gene. NSCLC and breast Ca cells were obtained from patients, and these were implanted in animal models, which were treated with vinorelbine. After tumor relapse, there was induction of enhanced angiogenesis and metastasis. These chemoresistant tumor cells were treated with the induced pluripotent stem cells, which were encoded with shRNA against GRP78.
Results: Post-treatment, the stem cells encoded with anti-GRP78 shRNA converted dicer into a small-interfering RNA molecule generating a long-lasting RNAi silencing effect of GRP78, which spread to adjacent tumor cells, inducing a gene-silencing bystander effect. Capillary growth into the tumors was blocked, whereas the angiogenic growth factors vascular endothelial growth factor and basic fibroblast growth factor were downregulated. Furthermore, the antiangiogenic enzyme PKG was upregulated, inhibiting β-catenin. Integration of endothelial precursor cells and tumor cells was blocked, inhibiting growth of mosaic blood vessels. This led to inhibition of tumor spread or metastasis, whereas the existing tumors died from lack of nutrients/oxygen and a waste disposal pathway. TEM exhibited induction of type I PCD or apoptosis in tumor cells, leading to a bystander killing effect. Thus, anti-GRP78 iPSCs circumvented vinorelbine-induced angiogenesis, and metastasis eradicating chemoresistant NSCLC and breast cancer cells.
Conclusions: Vinorelbine-induced angiogenesis and metastatic spread in NSCLC and breast Ca are circumvented with iPSCs encoded with anti-GRP78 shRNA, which induces apoptosis after a gene-silencing bystander effect.

Definition of the Immunologic Properties of Cancer Stem Cells Isolated From Human Glioblastoma

*Cristina Maccalli**, Ena Wang†, Tiziano Di Tomaso*, Stefania Mazzoleni‡, Gloria Sovena*, Soldano Ferrone§, Rossella Galli‡, Francesco Marincola†, Giorgio Parmiani*. **Molecular Oncology, Unit of Immuno-biotherapy of Melanoma and Solid Tumors;* ‡*Stem Cell Research Institute, San Raffaele Foundation Scientific Institute, Milan, Italy;* †*Transfusion Medicine, National Institutes of Health, Bethesda, MD;* §*Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

Background: The main objectives of our study were to characterize the immune profile of cancer stem cells (CSCs) isolated from glioblastoma multiforme (GBM) and to identify potential molecules for immunotherapeutic target for GBM patients.
Methods: We assessed by IF and cytofluorimetric or confocal microscopy analysis the immune profiles of 9 GBM CSC lines. Five of them also had parental autologous tumor cell lines in vitro cultured in the presence of fetal bovine serum (FBS). Moreover, we determined the efficiency of these CSC and FBS lines in eliciting T-cell – mediated anti-GBM activity by analyzing the cytokine release [interferon (IFN)-γ and interleukin (IL)-5] or the cytotoxic activity (CD107a mobilization) of in vitro-stimulated autologous lymphocytes.
Results: Both GBM CSCs and FBS tumor cells were found to be weakly positive or negative for the expression of major histocompatibility complex (MHC) class I or class II molecules, respectively. Moreover, we observed in these cell lines a defective expression of a large array of molecules involved in antigen processing and trafficking. Upregulation of MHC class I and antigen processing and trafficking molecules, in contrast to MHC class II molecules, was achieved after IFN-α or IFN-γ treatment of the cells. Along these lines, weak or no expression of NKG2D ligands (MICA/B or ULBPs) was detected by most GBM CSC and FBS lines. Interestingly, Survivin, COA-1, and SOX2 were efficiently expressed by both GBM CSC and FBS tumor lines. Moreover, these GBM lines released high levels of proangiogenic, chemotactic factors (MCP1, vascular endothelial growth factor, and Ang2) and cytokines (eg, IL-6, IL-8, and transforming growth factor-β). Transcriptome profile analysis revealed that 469 transcripts, including genes involved in immunologic pathways, were differentially expressed between CSC and FBS tumor cell lines; the functional significance of these data are currently being evaluated. At functional level, we found that in 1 GBM patient, IFN-γtreated CSCs could elicit T-cell mediated immune response specific for CSCs through stimulation of autologous peripheral blood mononuclear cells. Conversely, mostly Th2 subset-mediated immune responses were found in 3 other GBM patients.
Conclusions: Taken together, these results indicate that, although the antigenic profile of GBM CSCs still needs to be better defined, these cells represent low efficient antigenic source for eliciting T-cell-mediated immune responses in some GBM patients.

TARGETED THERAPEUTICS AND BIOLOGICAL THERAPY

In Vitro Anticancer Property of NBD Peptide Through Inhibition of Nuclear Factor-κB Activation

*Maria L. Ascierto**†, Giusy Gentilcore†, Carmela Gravina†, Andrea Worschech*, Zoltan Pos*, Richard Y. Wang*, Emiliana Natale† ‡, Marilena Capone†, Anna De Maio‡, Giuseppe Pirozzi†, Silvana De Martino†, Ena Wang*, Francesco Marincola*, Paolo A. Ascierto†. **National Institutes of Health, Bethesda, MD;* †*National Cancer Institute;* ‡*Federico II University, Naples, Italy.*

Introduction: The transcription factor nuclear factor (NF)-κB is an important regulator involved in inflammation and cell proliferation. Its activation requires the activity of an inhibitor of κB (IκB)-kinase (IKK) complex containing 2 kinases (IKKα and IKKβ) linked to the regulatory protein NEMO. The identification of a cell-permeable NBD peptide that blocks the association of NEMO with the IKK complex 1 has provided the possibility of selectively modulating the activation and NF-κB – dependent gene expression. Our earlier studies show that NBD peptide is able to block the activity of IKK complex, inducing apoptosis, in A375 melanoma cells 2. In this study, we investigate the anticancer property and induction of apoptosis of NBD peptide on several types of human and murine melanoma cell lines (397, M14, SK-Mel-2, PES 43, PES 47, COPA 159, and B-16) plus other nonmelanoma cells among the NCI-60 panel.
Method: Basal activation of the NF-κB signal transduction pathway in each cell line was measured by the secreted alkaline phosphate (SEAP) reporter gene assay, using the vector pTAL-SEAP as negative control and the vector pMet-Luc as positive control of the transfection. To determine the induction of apoptosis by wild type (wt) NBD, cells were plated at a density of 2×10^5/mL in 6-well plates, and on the following day different concentrations of wt NBD (50-100 μM) were added. Control cells were treated with the same concentration but using a mutant NBD peptide. At different incubation time points, the cells were collected and proapoptotic effects were determined by flow cytometry using the phycoerythrin-conjugated antihuman active caspase-3. To confirm the proapoptotic effect of wt NBD, PARP's cleavage-treated cells were determined by Western blot.
Results: The results obtained by SEAP reporter gene assay showed that all cells treated with the peptide have a basic activation of the NF-κB pathway. The induction of apoptosis in cells treated with wt NBD is shown by an increase in active caspase-3 in a dose-dependent (50 μM and 100 μM) and time-dependent (5 to 24 h) manner compared with the cells treated with mutant NBD. Apoptosis induced by wt NBD is also confirmed by PARP cleavage.
Conclusions: Our results show that specific blockade of NF-κB by the wt NBD peptide induces strong apoptosis in several cells. Our next aim is to confirm these results in vivo. This study provides further support for the hypothesis that selective inhibition of NF-κB activation can be an effective strategy for challenging melanoma and other cancers.

Soluble, High-Affinity T-cell Receptors as Cancer Therapeutics

*Rebecca Ashfield**, Katherine Adams*, Giovanna Bossi*, Daniel Williams*, Rebecca Dennis*, Samantha Paston*, Jane Harper*, Namir Hassan*, Emmet McCormack†, Bent Jakobsen*. **Immunocore Ltd, Abingdon, United Kingdom; †Medical Faculty, University of Bergen, Bergen, Norway.*

T-cell receptors (TCRs) are able to recognize cancer-associated peptide epitopes presented on the surface of tumor cells by class I human leukocyte antigen (HLA) molecules. Many cancer patients, however, are unable to mount an effective antitumor T-cell response, either as a result of low T-cell avidity or of the induction of tumor escape mechanisms resulting in T-cell inhibition or anergy. To generate potent tumor-targeting agents, we engineered soluble T-cell receptors (sTCRs), which retain the native αβ structure, and used phage display technology to produce high-affinity variants with picomolar affinities. Specifically, high-affinity sTCRs have been generated, which recognize 3 cancer-associated epitopes: NY-ESO157-165, Melan-A/MART 126-35, and gp100 280-288, all presented by HLA-A2. The sTCRs specifically target HLA-A2+, antigen+melanoma cells in vitro and in vivo.

With the aim of generating therapeutic proteins for cancer, we fused high-affinity sTCR molecules to an scFv antibody domain specific for CD3. These fusion proteins simultaneously target HLA peptide complexes on the surface of tumor cells, and activate adjacent $CD3^+$ T cells regardless of their antigen specificity. Such "redirected" T cells kill tumor cells expressing low levels of target antigen (< 100 molecules/cell). We have shown that sTCR-antiCD3 fusions (a) kill melanoma cells expressing physiologic levels of epitope in vitro, (b) are more potent than a T-cell clone, and (c) demonstrate antitumour activity in vivo. We are currently planning to carry out a clinical trial in cancer patients with an sTCR-antiCD3 fusion protein.

Elevated Soluble Major Histocompatibility Complex Class I Chain-related Protein A Predicts Nonresponsiveness to Cytotoxic T Lymphocyte Antigen-4 Blockade

*Keith S. Bahjat**†, Alan J. Korman†. **Cancer Research, Earle A. Chiles Research Institute, Portland, OR; †Discovery Research, Medarex, Milpitas, CA.*

NKG2D on cytolytic natural killer (NK) and T cells plays an essential role in the recognition and destruction of transformed cells. The NKG2D ligand MICA (major histocompatibility complex class I chain-related protein A) is expressed by a variety of human tumors. The soluble form of MICA (sMICA), which acts as an inhibitor of NK cells and cytolytic effector cells, has been reported in the serum of patients with malignant disease. We questioned how the presence of potentially suppressive sMICA might impact the efficacy of immunotherapy such as cytotoxic T lymphocyte (CTLA)-4 blockade. Multiple clinical trials treating metastatic melanoma with anti−CTLA-4 have shown objective responses and prolonged overall survival (OS). We measured sMICA in the serum of melanoma patients before and after receiving a regimen of anti−CTLA-4 (ipilimumab). Sera were analyzed from 89 patients from BMS Study CA184007, which investigated the response and OS of patients who received 10 mg/kg of ipilimumab (Q3W × 4; followed by 10 mg/kg every 12 wk) with and without oral budesonide (n = 115). CA184007 showed an objective response rate of 13.9% at 12 weeks, with an 18-month survival rate of 49.4%. These melanoma patients showed significantly elevated levels of sMICA relative to normal donors ($P < 0.001$). In addition, patients with detectable levels of sMICA (> 80 pg/mL) before therapy were more likely to be nonresponsive to ipilimumab treatment ($P < 0.01$) as measured by objective RECIST criteria. Furthermore, patients with detectable sMICA showed decreased OS ($P < 0.009$), with an 18-month survival rate of 35.4% for the pretreatment sMICA-positive group and 64.8% for the pretreatment sMICA-negative group. These data suggest that sMICA might limit the efficacy of tumor-specific T cells during CTLA-4 blockade, and that inhibiting sMICA may potentiate CTLA-4 blockade and other immunotherapies as well.

Reengineering Clinical-grade Dendritic Cells by Glucocorticoid-induced Leucine Zipper Silencing to Develop a Highly Potent Cancer Vaccine

Dominique Cathelin, Özcan Met, Inge Marie Svane. *Center for Cancer Immune Therapy (CCIT), Herlev, Denmark.*

Considering the number of studies on dendritic cell (DC)-based cancer vaccines over this decade, this immune cell population seems to be one of the most important immune regulators. DCs are differentiable in vitro from patient-circulating precursors, and are easy to manage before reinjection. The maturation cocktail including interleukin (IL)-6, IL-1β, tumor necrosis factor-α, and prostaglandin E_2 was considered the gold standard to induce DC maturation before its use for cancer immune therapy. Nevertheless, although this maturation cocktail induces an increased expression of several activation markers such as CD83, the costimulation molecules CD80, CD86, and CD40, and chemokine receptor involved in the DC homing in lymph nodes CCR7, the DC immune stimulatory function in vivo contrast with this mature phenotype and complete responses in cancer patients treated with DC-based vaccines remain rare.

In this study, we showed that after a 48-hour treatment of DCs with the cocktail IL-6, IL-1β, tumor necrosis factor-α, and prostaglandin E_2, the mature phenotype is associated with the expression of several immunosuppressive regulators including the indoleamine 2,3-dioxygenase, B7-H1, and the glucocorticoid-induced leucine zipper (GILZ). In addition, we found a high secretion level of IL-10. It has already been shown that the overexpression of the transcription factor GILZ may be associated with expression of B7-H1 and secretion of IL-10 by myeloid cells. In ongoing experiments, we target GILZ in the clinical-grade DCs by small-interfering RNA to silence their immunosuppressive activities and to develop a highly immunogenic cancer vaccine.

Multifunctional Nanoparticles: A Nonviral Vector for Tumor-targeted Delivery of Therapeutic Small-interfering RNA and Peptide Nucleic Acid Oligonucleotides

*Christopher J. Cheng**, Mark Saltzman†. **Molecular Biophysics and Biochemistry; †Biomedical Engineering, Yale University, New Haven, CT.*

The main limitation of effectively using antisense agents to post-transcriptionally regulate therapeutic gene targets is suboptimal delivery. We designed biodegradable nanoparticles that safely and efficiently deliver both small-interfering RNAs (siRNA) and peptide nucleic acids (PNA) to human epithelial cancer cells. These nanoparticles protect encapsulated oligonucleotides from nucleases, prolong in vivo circulation time, and passively target leaky tumor vasculature. Furthermore, we designed a novel surface attachment method to endow the synthetic nanoparticles with ligands that impart virus-like delivery capabilities. To enhance the efficacy of this delivery system, we coated the nanoparticles with functional ligands that mediate specific cell targeting, facilitate cell entry, and promote endosome escape. In culture, these ligand-functionalized nanoparticles were preferentially targeted to cells that overexpress cancer-associated receptors. Internalized nanoparticles were able to release siRNAs or PNAs that suppress the expression of luciferase and the chemotherapeutic targets, thymidylate synthase, and Mdm2. In contrast, nonfunctionalized nanoparticles showed limited cellular uptake and less efficient inhibition of gene expression. We developed a targeted delivery vector that effectively elicits therapeutic RNAi, and presents a novel platform for the controlled transport of hard-to-deliver

PNAs. Modification of these nanoparticles to display different ligands presents a universally adaptable oligonucleotide delivery system that can be fine-tuned to overcome physiologic and cellular barriers that limit the efficacy of these potent therapeutic agents.

IMMUNOPOTENT C-REACTIVE PROTEIN Enhances the Activity of Cisplatin and Prevents its Side Effects

Moisés A. Franco Molina, Edgar Mendoza-Gamboa, Clara Isaza Brando, Pablo Zapata Benavides, Paloma Castillo Tello, Diana Zamora Avila, Diana Miranda Hernández, Crystel Sierra Rivera, Magda Vera Garcíat, Reyes Tamez Guerra, Cristina Rodríguez Padilla. *Inmunologia y Virología, Facultad de Ciencias Biológicas de la UANL, San NIcólas de los Garza, Mexico*.

Cisplatin (cis-diamminedichloroplatinum II) is one of the most effective chemotherapeutic agents used in the treatment of a variety of human solid tumors. However, its clinical use is limited owing to severe toxicity. IMMUNOPOTENT C-REACTIVE PROTEIN (CRP) is a mixture of low-molecular weight substances (cutoff of 10-12 kd) that come from the dialysis of disintegrated bovine spleens in sterile water. IMMUNOPOTENT CRP had cytotoxic effects in several cancer cell lines by apoptosis and modulated several transcription factors involved in cellular proliferation, in a phase I study in lung cancer patients maintaining immunologic protection against chemotherapeutic side effects.

Aims: To determine whether IMMUNOPOTENT CRP mixed with cisplatin affects the cell viability and tumor growth of B16F10 melanoma.

Methods: B16F10 cells and mice challenged with B16F10 cells were treated with different concentrations of cisplatin and IMMUNOPOTENT CRP + cisplatin. Thereafter, these were evaluated by MTT, Greiss reactive, TUNEL, caspase-3, senescence, and VEGF assays and parameters related to survival, behavior analysis, and tumor weight.

Results: Cisplatin and IMMUNOPOTENT CRP + cisplatin decreased the viability of B16F10 cells through apoptosis. IMMUNOPOTENT CRP + cisplatin increased the expression of caspase-3 and the senescence induced by cisplatin alone; vascular endothelial growth factor production was decreased both in vitro and in vivo. Furthermore, treatment prevented metastasis, delayed the appearance of tumors, improved behavior, decreased tumor weight, and increased the survival of tumor-bearing mice.

Discussion: These observations suggest that IMMUNOPOTENT CRP can be used in combination with cisplatin to improve its chemotherapeutic activity and prevent its collateral effects (Table 1).

The Study of mIR-15A and mIR-16-1 Enhancement of the Sensitivity of Raji Cells to Cytarabine

Dongmei He, Qin Chen, Feiyan Zou. *Jinan University, Guangzhou, China*.

Aim: To determine whether miR-15a and miR-16-1 can enhance the sensitivity of Raji cells to cytarabine (Ara-C).

Methods: The miR-15a and miR-16-1 oligonucleotides were synthesized transfecting the miR-15a an miR-16-1 oligonucleotides Raji cells with Lipofectamine™ 2000, and then respectively combined with Ara-C. IC50 were detected by CCK8 assay. The growth inhibitory effect of Raji cells was measured by trypan blue dye exclusion method and CCK8 assay. The apoptotic cells were observed by Hoechst dyeing; Annexin V/propidium iodide double dyeing method was used to detect the cell apoptotic rate by flow cytometry (FCM).

Results: miR-15a or miR-16-1 oligonucleotides combined with Ara-C significantly decreased the IC50 of Ara-C. Trypan blue dyeing exclusion method and CCK8 assay showed that miR-15a/16-1 plus Ara-C obviously decreased the cell growth more than miR-15a/16-1 alone, Ara-C alone, or random sequence plus Ara-C. Plenty of apoptotic cells can be observed with Hoechst dyeing. Annexin V/propidium iodide double dyeing assays by FCM indicated that the cell apoptotic rates in the earlier period and advanced stage of miR-15a plus the Ara-C group were obviously higher than miR-15a/16-1 alone, Ara-C alone, or random sequence plus Ara-C.

Conclusions: miR-15a and miR-16-1 oligonucleotides can enhance the sensitivity of Raji cells to Ara-C.

The Study of Pre-MIR-15A Inducing Apoptosis on Lymphomatic Cells

Dongmei He. Jinan University, Guangzhou, China.

Eukaryotic expression vector of the microRNA-15a (miR-15a) precursor (pre-miR-15a) was constructed and the effects of miR-15a on the growth of the lymphoma Raji cell line were investigated.

Methods: A pair of oligonucleotides for pre-miR-15a expression were designed and chemically synthesized. Annealed oligonucleotides were inserted into the pGCSIL-GFP vector under U6 promoter to construct the pre-miR-15a expression plasmid. The oligonucleotide with a scrambled sequence, which was used as a negative control, was also constructed into a pGCSIL-GFP vector. The recombinant expression vector was identified by polymerase chain reaction (PCR) and sequencing. Pre-miR-15as expressing plasmid and negative control plasmid were all transfected into Raji cells with lipofectamine 2000. Quantitative reverse-transcription (qRT)-PCR was employed to explore the expression of miR-15a. Bcl-2 protein expression was detected by indirect immunofluorescence;

TABLE 1. (Molina) Mean Time of Tumor Appearance, Death, and Survival of C57BL/6 Mice Inoculated With 2×10^5 of B16F10 Melanoma Cells and Treated With Cisplatin and Immunopotent CRP+Cisplatin

Treatments	Time of Tumor Cell Inoculation and Treatment	Mean Time to Appearance of Tumor (Days ± SD)	Mice With Visible Tumor Mass (%)	Mean Time of Death Mice (Days ± SD)	Mice Death (%)	Ovelife (d)	Mice Survival (%)
Mice survival (%)	0	0	0	0	0	0	0
RI	0	13.6 ± 2.8	100	29.2 ± 4.2	100	0	0
Cisplatin 8 mg/kg	0	20 ± 0.8	50	54.5 ± 4.9	50	25.3 ± 0.37	50
Immunopotent CRP 1 U + cisplatin 8 mg/kg	0	20 ± 2	50	54.9 ± 5.1	50	25.7 ± 0.49	50
Immunopotent CRP 3 U + cisplatin 8 mg/kg	0	21.5 ± 4.9	50	57.5 ± 3.5	50	28.3 ± 0.25	50

CRP indicates C-reactive protein.

semiquantitative RT-PCR detected the expression level of Bcl-2 mRNA; the apoptotic cells were observed by Hoechst dyeing; and propidium iodide dyeing was used to detect the cell apoptotic rate by flow cytometry. The growth inhibitory effect of Raji cells was measured by the CCK8 assay.
Results: Identification by PCR and sequencing showed that the insertion sequence was correct. The expression level of miR-15a was noticeably increased in Raji cells transfected with the pre-miR-15a plasmid, which was much higher than the blank and the negative control groups. After Raji cells were transfected with the pre-miR-15a expressing plasmid for 48 hours, Bcl-2 protein expression levels decreased considerably, which were obviously different compared with the negative control and blank groups ($P < 0.05$). The CCK8 assay showed that transfection of pre-miR-15a expressing plasmid decreased cell growth at 24, 48, and 72 hours posttransfection. After Raji cells were transfected with pre-miR-15a expressing plasmid, apoptotic cells can be seen with Hoechst dyeing and the cell apoptotic rate was considerably higher than in the blank and negative control groups.
Conclusion: Pre-miR-15a can induce apoptosis of Raji cells.

Targeting JAK-STAT Signaling for Cancer Therapy

*Michael Hedvat**, Dennis Huszar†, Michael Zinda†, Hua Yu*, Richard Jove*. **Beckman Research Institute, City of Hope Cancer Center, Los Angeles, CA; †Cancer Bioscience, AstraZeneca, Boston, MA.*

The Signal Transducer and Activator of Transcription (STAT) family of proteins comprise transcription factors that mediate cytokine and growth factor responses. Persistent activation of 1 STAT family member, Stat3, is oncogenic and prevalent in a wide variety of human cancers, including solid and hematologic tumors. Stat3 activation promotes the growth and survival of human cancer cells by inducing constitutive expression of genes that encode antiapoptotic proteins, cell cycle regulators, and angiogenic factors. Moreover, Stat3 has an important role in autocrine and paracrine cytokine loops driving many human cancers. Stat3 activation by cytokines such as interleukin-6 (IL-6) is mediated through the Janus family kinases (JAK) including Jak2. These observations provide a molecular basis for persistent Stat3 activation in solid tumors, and highlights Jak2 kinase as a potential target for cancer therapy. Using novel small-molecule Jak2 inhibitors, we show a predominant role of Jak2 in mediating basal and cytokine-induced Stat3 activation in solid human tumor cell lines. Blockade of Stat3 activation with Jak2 inhibitors is associated with the abrogation of Stat3 nuclear translocation and tumorigenesis. In IL-6-expressing tumor cells, Jak2 is a key regulator of IL-6 autocrine signaling, thereby promoting the growth and survival of these cells. Treatment of tumor-bearing mice with the Jak2 inhibitor suppresses the growth of human prostate, ovarian, and breast cancer xenografts harboring Stat3 activity. Our data support a central role of Jak2 in Stat3-dependent oncogenesis, and provide a rationale for targeting Jak2 in human solid tumors with persistent Stat3 activity.

Investigation of Anticancer Effects of Boric Acid in Vitro

Hakan Istanbulluoglu, Recai Ogur, Mahmut Ilker Yilmaz, Omer Faruk Tekbas. *Gulhane Military Medical Academy, Ankara, Turkey.*

Summary: We investigated the anticancer effects of boric acid in breast cancer in vitro. The inhibition was investigated by examining the impact of boric acid on the cell viability by MTT test and apoptosis.
Background/Aim: After lung cancer, breast cancer is the second leading cause of cancer deaths in women today. Some studies have identified boron as a chemopreventative agent in a few types of cancer such as prostate. The effect of boric acid in breast cancer has not been examined in detail till date. The present objective was to investigate boric acid-induced anticancer effect in the MCF-7 human breast cancer cell line.
Materials and Methods: Breast cancer cells (MCF-7) were cultured in MEM-EAGLE supplemented with 10% fetal bovine serum, penicillin/streptomycin (1%), and L-glutamine (1%). The cells were cultured at 37.8°C and 5% CO_2. Boric acid (0.1 g) was extracted by using 10 mL of ion exchange water and added to human breast cancer cell line, MCF7. At the end of the incubation period, the plates were analyzed by using the standard MTT cell viability test. Briefly, plated in 96-well plates with MEM-EAGLE, FBS, penicillin/streptomycin and L-glutamine contain boric acid. After culturing for 24 hours at 36.5°C, and CO_2 5%, 10 μL of MTT were added to each well and incubated for 4 hours. After dissolving with isopropanol, the absorbance was read at 570 nm. Double staining was performed to quantify the number of apoptotic cells in culture on the basis of the scoring of apoptotic cell nuclei by using MCF7 breast cancer cells. After treating with boric acid for 72 hours, period cells were collected, then washed and stained with Hoechst dye 3342 (2 mg/mL), propidium iodide (1 mg/mL), and DNAse-free RNAse (100 mg/mL) for 15 minutes at room temperature. After that, 10 to 50 mL of the cell suspension was smeared on the slide and cover slip for examination by fluorescence microscopy.
Results: The extract of boric acid showed a powerful and statistically significant anticancer effect on MCF-7 human breast cancer cells (about a decrease of 41% in cell viability). This inhibition, in the MCF-7 cell line, was also observed with the aid of MTT conversion analysis. Boric acid-induced apoptosis was confirmed by morphologic evaluation using fluorescence microscopy. The nuclei of normal or apoptotic cells were stained blue by the Hoechst dye. Necrotic cells were stained red by propidium iodide.
Conclusions: As a result of our MTT analysis, boric acid is confirmed to have an anticancer effect on the MCF-7 human breast cancer cell line in vitro. Future investigations are needed to elucidate the mechanism of boron's effect on proliferation, prevention, and control of breast cancer.

Targeted "Hybrid Peptide" That Binds Transferrin Receptor and Disintegrates Cancer Cell Membrane

Megumi Kawahmoto, Tomohisa Horibe, Masayuki Kohno, Koji Kawakami. *Pharmacoepidemiology, Kyoto University, Kyoto, Japan.*

Transferrin receptor (TfR) is a cell membrane-associated glycoprotein involved in the cellular uptake of iron and the regulation of cell growth. Recent studies have shown the elevated expression levels of TfR on cancer cells compared with normal cells. The elevated expression levels of this receptor in malignancies, which is the accessible extracellular protein, can be a fascinating target for the treatment of cancer. We have recently designed a novel type of immunotoxin, targeted "hybrid peptide," which is a chemically synthesized peptide composed of target-binding peptide and lytic peptide containing cationic-rich amino acid components that disintegrates the cell membrane for the killing of cancer cells. The lytic peptide is newly designed to induce rapid killing of cancer cells due to conformational change.
In this study, we designed TfR-binding peptide connected with this novel lytic peptide and assessed the cytotoxic activity in vitro.
Cytotoxic analysis using TfR-lytic peptide demonstrated that this peptide has low IC50 (the peptide concentration inducing 50% inhibition of control cell growth) in various cancer cell lines. On the other hand, the TfR-lytic peptide is less toxic to normal cell lines. The cytotoxic activity is dependent on the expression levels of TfR. Competitive assay using TfR antibody or knock down of this receptor by siRNA also indicated that TfR-lytic peptide is specific to TfR. In addition, it was revealed that the TfR-lytic peptide can penetrate the cell membrane to make pores on T47D breast cancer cell surface subsequently killing the cells within 10 minutes. We then performed apoptosis assay assessed by Annexin-V binding, caspase activity, and JC-1 staining to assess the change in mitochondrial membrane potential, and found that the TfR-lytic peptide induces approximately 80% of apoptotic cell death of T47D cells.
In conclusion, TfR-lytic peptide might provide a potent and selective anticancer therapy for patients.

Potent, Orally Active Hydroxylamidine Inhibitors of Indoleamine-2,3-dioxygenase (IDO) Suppress Growth of IDO-expressing Tumors Through Systemic Inhibition of Tryptophan Catabolism

Holly K. Koblish, Michael J. Hansbury, Kevin J. Bowman, Gengjie Yang, Richard B. Sparks, Eddy W. Yue, Andrew P. Combs, Peggy A. Scherle, Kris Vaddi, Jordan S. Fridman. *Incyte Corporation, Wilmington, DE.*

Several mechanisms prevent immune responses to malignant tumors; among them are inefficient antigen presentation and active immune suppression. Expression of indoleamine-2,3-dioxygenase (IDO; IDO1), a rate-limiting enzyme in the catabolism of tryptophan (Trp) into kynurenine (Kyn) contributes to this immune evasion by restricting the activity of lymphocytes. Single oral doses of INCB023843 or INCB024360, hydroxylamidine small-molecule inhibitors of IDO1, result in efficient suppression of Trp catabolism in the plasma of treated mice to levels seen in IDO1-deficient mice. These hydroxylamidines potently suppress Trp catabolism in vitro in multiple cancer cell lines and in vivo in tumors and their draining lymph nodes (TDLNs). Repeated administration of these IDO1 inhibitors was well tolerated and impedes tumor growth in a dose-dependent and lymphocyte-dependent manner. Substantiating the fundamental role of tumor cell-derived IDO expression, these hydroxylamidines control the growth of IDO-expressing tumors in IDO1-deficient mice. The increased responsiveness of lymphocytes and the reduction in the numbers of T regulatory cells found in tumors and TDLNs are likely to contribute to tumor growth control. INCB024360, a potent IDO1 inhibitor with desirable pharmaceutical properties, is poised to enter clinical testing.

STAT3-specific Small-molecule Curcumin Analogs FLLL32 and FLLL62 Induce Apoptosis and Enhance the Direct Antitumor Effect of Interferons

Matthew A. Bill, James R. Fuchs, Chenglong Li, Eric B. Schwartz, Jiayuh Lin, Dale G. Hoyt, Benjamin Foor, Dan Otterson, Jennifer Yui, William E. Carson, Pui-Kai Li, *Gregory B. Lesinski. The Ohio State University Comprehensive Cancer Center, Columbus, OH.*

STAT3 is critical for promoting an oncogenic, metastatic, and drug-resistant phenotype in several types of cancer including renal cell carcinoma (RCC). One promising lead compound for STAT3 inhibition is the natural product, curcumin. Curcumin has chemopreventative and chemotherapeutic properties in numerous cancer models and can inhibit a variety of cellular targets including STAT3. Recent studies from our group have also indicated that curcumin can adversely affect the responsiveness of immune cells to clinically relevant cytokines [interferon (IFN)-α, IFN-γ, interleukin (IL)-2, IL-12] that possess antitumor properties. To overcome this lack of selectivity, we used molecular modeling to synthesize numerous curcumin analogs. The goal of these studies is to improve upon the pharmacologic profile of the natural product (ie, selectivity, bioavailability, and stability) and generate compounds that, unlike curcumin, do not antagonize the action of IFNs or ILs. Computational chemistry and structure-activity relationship studies have predicted that the diketo-tautomer but not the enol-tautomer of curcumin can inhibit STAT3 dimerization. Therefore, we designed curcumin derivatives locked into the diketo form with the goal of enhancing the specificity for STAT3 as a molecular target. FLLL32 and FLLL62 are structurally distinct curcumin analogs that specifically target the SH2 domain of the STAT3 protein, and inhibit its phosphorylation and dimerization. We hypothesized that FLLL32 and FLLL62 (FLLL32/62) would induce apoptosis in RCCs and display specificity for STAT3. In vitro studies revealed that FLLL32/62 reduced phosphorylated STAT3 (pSTAT3), and induced apoptosis at micromolar amounts in the SK-RC-45, SK-RC-54, ACHN, and SK-RC-13 human RCC cell lines as determined by Annexin V/propidium iodide staining. FLLL32-induced apoptosis was confirmed by immunoblot analysis of caspase-3 processing and poly-ADP ribose polymerase cleavage. The expression of STAT3-regulated cyclin D1 protein was also reduced in response to FLLL32/62. These curcumin analogs were specific in that they did not interact with STAT1 protein. Pretreatment of melanoma cells with FLLL32/62 did not inhibit IFN-α – induced pSTAT1 or downstream STAT1-mediated gene expression as determined by real-time polymerase chain reaction. Finally, concurrent treatment of RCC cell lines with FLLL32 and IFN-α or IFN-γ led to further enhancement of apoptosis. These data support further investigation of FLLL32/62 as lead compounds of RCC therapy and suggest that STAT3 inhibition represents a rational approach for enhancing the direct antitumor actions of IFNs.

Small-molecule Curcumin Analogs Induce Apoptosis in Human Melanoma Cells via STAT3 Inhibition but Do Not Alter the Cellular Response to Immunotherapeutic Cytokines

Gregory B. Lesinski, Matthew A. Bill, James R. Fuchs, Chenglong Li, Courtney Bakan, Don M. Benson, Eric B. Schwartz, Jiayuh Lin, Dale G. Hoyt, Stacey L. Fossey, Gregory S. Young, William E. Carson, Pui-Kai Li. *The Ohio State University Comprehensive Cancer Center, Columbus, OH.*

Curcumin has antitumor activity in numerous experimental cancer models and has been shown to inhibit a variety of cellular targets including STAT3. However, recent studies from our laboratory indicated that curcumin adversely affects the responsiveness of immune cells to cytokines with antitumor properties [interferon (IFN)-α, IFN-γ, interleukin (IL)-2, IL-12]. To overcome this lack of selectivity, we synthesized and evaluated curcumin analogs predicted to target STAT3 via molecular modeling and cell-based assays. It was hypothesized that these curcumin analogs could serve as potent, STAT3-specific inhibitors that induce melanoma cell apoptosis but do not antagonize the action of IFNs or ILs. FLLL32 is a small-molecule inhibitor of STAT3 phosphorylation and dimerization that approximates curcumin when locked into its diketone tautomeric form. Time course and dose-response studies revealed that FLLL32 reduced phosphorylated STAT3 (pSTAT3), STAT3-DNA binding and induced apoptosis at micromolar amounts in multiple human metastatic, vertical, and radial growth phase melanoma cell lines (IC50 range = 1.3 to 2.1 μM at 48 h). FLLL32 induced processing of caspase-3, caspase-8 and caspase-9 proteins, reduced cyclin D1 (a STAT3-regulated protein), and decreased mitochondrial membrane potential. Cell death induced by FLLL32 was caspase-dependent as the pan-caspase inhibitor (Z-VAD-FMK) abrogated the proapoptotic effects of this compound. FLLL32 was specific in that it did not interact with STAT1 protein. Pretreatment of melanoma cells with FLLL32 did not inhibit IFN-γ – induced pSTAT1 or downstream STAT1-mediated gene expression (IFN-regulatory factor-1) as determined by real time polymerase chain reaction. As the STAT3 function in immune cells can promote tolerance to tumors, we evaluated whether FLLL32 would affect the responsiveness of peripheral blood mononuclear cells (PBMCs) to stimulation with cytokines that play a role in tumor progression (IL-6) or immunosurveillance (IFN-γ, IL-2). Pretreatment with FLLL32 eliminated basal and IL-6 – induced pSTAT3 in PBMCs from healthy donors. In contrast, FLLL32 pretreatment did not adversely affect IFN-γ – induced pSTAT1 or IRF1 transcription in PBMCs. Similarly, FLLL32 pretreatment did not adversely affect IL-2 – induced STAT5 phosphorylation. Importantly, treatment of PBMCs or NK cells with FLLL32 did not decrease viability or granzyme B and IFN-γ production when cultured with K562 targets. These data support further investigation of FLLL32 as a lead compound for STAT3 inhibition and therapy of melanoma and other malignancies.

A Novel Small Molecule Inhibits STAT3 Phosphorylation and Activities and Exhibits Potent Growth Suppressive Activity in Human Cancer Cells

*Li Lin**†, Brian Hutzen*‡, Sarah Ball*‡, Mingxin Zuo*, Stephanie DeAngelis*, Elizabeth Foust*, Pui-Kai Li§, Chenglong Li§, Jiayuh Lin*‡. **Department of Pediatrics, College of Medicine; ‡Molecular, Cellular, and Developmental Biology Graduate Program; §Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH; †Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.*

Constitutive activation of Signal Transducers and Activators of Transcription 3 (STAT3) signaling is frequently detected in many types of cancer, promoting its emergence as a promising molecular target for cancer treatment. Inhibiting constitutive STAT3 signaling represents a potential therapeutic approach. We used a structure-based design to develop a nonpeptide, cell-permeable, small molecule, termed as LLL12, which targets STAT3. LLL12 was found to inhibit STAT3 phosphorylation (Tyr705) and induce apoptosis as indicated by the increases of cleaved caspase-3 and poly-ADP ribose polymerase in various human breast, pancreatic, colon, liver cancer cells, multiple myeloma, and glioblastoma cell lines expressing elevated levels of STAT3 phosphorylation. However, LLL12 does not induce apoptosis in several normal human cell types that lack constitutive STAT3 signaling. LLL12 also can inhibit STAT3 phosphorylation induced by interleukin-6 in MDA-MB-453 breast cancer cells without endogenous phosphorylated-STAT3. The inhibition of STAT3 signaling by LLL12 was confirmed by the inhibition of STAT3 DNA binding activity. LLL12 also inhibited STAT3-dependent transcriptional luciferase activity in a dose-dependent manner. Downstream targets of STAT3, cyclin D1, Bcl-2, and survivin were also downregulated by LLL12 at both protein and mRNA levels. LLL12 is a potent inhibitor of cell proliferation, IC50 values are lower than previously characterized JAK2 inhibitor WP1066 and STAT3 SH2 inhibitor S3I-201 in all the independent cancer cell lines that we tested. LLL12 also inhibits colony formation, cell migration and works synergistically with doxorubicin and gemcitabine in the breast and pancreatic cancer cell lines. Furthermore, LLL12 was shown to inhibit tumor growth in U87 glioblastoma mouse xenografts. Our results suggest that the novel small-molecule STAT3 inhibitor, LLL12, may be a potential therapeutic agent for human cancer cells expressing constitutive STAT3 signaling.

Chemoimmunotherapy Using Polysaccharide Krestin and Paclitaxel is Superior to Standard Neoadjuvant Therapy in a Mouse Model of Locally Advanced Neu-mediated Breast Cancer

Hailing Lu, Yi Yang, Ekram Gad, Mary L. Disis. *Tumor Vaccine Group, University of Washington, Seattle, WA.*

Locally advanced breast cancer poses a significant clinical challenge because it is associated with a high relapse rate and poor overall survival. Current standard neoadjuvant therapy will only achieve pathologic complete response in about 50% of the patients. It is now recognized that chemotherapy has an immunogenic effect due to the release of multiple tumor antigens from dying tumor cells. We hypothesize that a ''danger signal'' that can enhance the uptake and presentation of tumor antigens may potentiate the immunogenic effect of chemotherapy and result in optimal tumor destruction in the neoadjuvant setting. We recently found that polysaccharide krestin (PSK), an extract from medicinal mushroom, has potent TLR2 agonist activity and stimulates the function of dendritic cells, T cells, and natural killer cells. We hypothesize that combining PSK and chemotherapy may increase pathologic complete response. Tumor-bearing neu-transgenic mice were treated with PSK (100 mg/kg, oral gavage, 3 times/wk), paclitaxel (15 mg/kg, intravenous, 3 times/wk), PSK + paclitaxel, or control PBS. PSK in addition to paclitaxel significantly inhibited tumor growth compared with paclitaxel alone. Furthermore, we show that PSK significantly potentiates antibody-dependent cell-mediated cytotoxicity mediated by trastuzumab. Altogether, these data support the integration of PSK into standard neoadjuvant therapies for HER2+/ER− locally advanced breast cancers.

A Clinical Study Combining Multiple Immune Modulators and an Antigen-Presenting Cell-targeted hCGβ Vaccine (CDX-1307)

*Michael Morse**, Robert Chapman†, John Powderly‡, Ding Wang†, Tibor Keler§, Lizhen He§, Venky Ramikrisha§, Laura Vitale§, Timothy Clay*, Jennifer Green§, Thomas Davis§. **Duke University Medical Center, Durham; ‡Carolina BioOncology Institute, Huntersville, NC; †Henry Ford Health System, Detroit, MI; §Celldex Therapeutics, Inc, Needham, MA.*

Background: Effective immunization requires efficient antigen processing and presentation by appropriately activated antigen-presenting cells. Preclinical studies have indicated that combining specific TLR agonists enhances immune responses and antitumor activity. We have developed a regimen that combines granulocyte macrophage-colony-stimulating factor, the TLR3 agonist Poly-ICLC (PIC) (Oncovir), and the TLR7/8 agonist Resiquimod (R) (3M) with CDX-1307, an antigen-presenting cell-targeted vaccine composed of a mannose receptor-specific monoclonal antibody (B11) fused with hCGβ, a tumor antigen correlated with advanced disease and poor prognosis in a number of common epithelial cancers.

Methods: Two phase I studies investigate intradermal (ID) versus systemic (IV) administration of CDX-1307. Patients with advanced cancers known to frequently express hCGβ receive 4 biweekly vaccinations of CDX-1307, alone or with immunostimulants including GM, PIC, R.

Results: Seventy-seven patients (cancer types: colorectal = 36, breast = 27, pancreatic = 10, bladder/ureteral = 2, ovarian = 1, or testicular = 1) were treated with CDX-1307 [0.3-2.5 mg ID (n = 47) or 1.0-30 mg IV (n = 30)], either alone (Group A; n = 34), or concurrent with GM ± single TLR agonist (group B; n = 34) or GM + dual TLR agonist (group C; n = 9). Toxicities were generally mild to moderate with no DLT. For the ID patients in groups A, B, and C, local reactions occurred in 28%, 75%, and 100%, and were of grade 2 severity in 0%, 0%, and 44%. Anti-hCGβ IgG responses developed in 19%, 47%, and 75% of the patients in groups A, B, and C, based on samples analyzed to date. TLR agonists enhanced the IgG titers (> 200,000 in 1 patient). In some patients, humoral responses developed despite high-circulating hCGβ and/or correlated with hCGβ decreases. To date, 9 patients with multiple histologies experienced stable disease for a range of 2.3+ to 7.2+ months. Humoral responses developed in 56% of those with stable disease versus 31% of those with progressive disease. Cell-mediated immune response analysis is ongoing.

Conclusions: CDX-1307 in heavily pretreated advanced cancer patients is well tolerated and immunogenic. A combination with multiple TLR agonists results in robust immune responses with increased local reactogenicity and may prime for antitumor activity. Phase II studies will examine clinical activity of this regimen in newly diagnosed patients, who tend to be more immunologically responsive and have the potential for altered disease course due to early immunologic intervention.

Arsenic Trioxide Induces Apoptosis in NB-4, an Acute Promyelocytic Leukemia Cell Line, Through Suppression of Nuclear Factor-κB-mediated Induction of Telomerase and Survivin

*Majid Momeny**, Seyed H. Ghaffari*, Majid Zakidizaji†, Davoud Bashash*, Ardeshir Ghavamzadeh*, Kamran Alimoghaddam*. **Hematology, Oncology and Bone Marrow Transplantation Research Center, Shariati Hospital; †Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran.*

This study intended to investigate the effects of arsenic trioxide (ATO) on human acute promyelocytic leukemia NB-4 cells. Cell

morphology, growth and viability assessments, microculture tetrazolium test, fluorescence microscopy and Annexin-V assessments of apoptosis, telomerase activity assay, assessment of telomere length, cell-based nuclear factor (NF)-κB activation assay, and real-time reverse transcription-polymerase chain reaction were used to appraise the effects of ATO on cell morphology, viability, and proliferation, induction of programmed cell death, telomerase enzymatic levels, telomere length, and NF-κB activation in NB-4 cells. ATO treatment caused nuclear shrinkage and membrane blebbing, suppressed cell growth, viability and metabolic activity, exerted apoptosis, hindered telomerase activity, shortened telomere length, and a dampened NF-κB activation module. Moreover, ATO exposure was associated with transcriptional repressions of human telomerase reverse transcriptase (hTERT), PinX1, survivin, and c-Myc along with an expressive enhancement in p73 message. These issues indicate for the first time that ATO might preempt cell growth and proliferation in NB-4 cells through the suppression of NF-κB – dependent stimulation of telomerase and survivin. Moreover, these outcomes imply that telomerase inhibition and telomere shortening may play a major role in ATO-induced cell death. On aggregate, the variegated suppressive effects of ATO on telomerase expression in this study propose the efficacy of ATO as a potent telomerase inhibitor.

Small-molecule Inhibitors of the Indoleamine 2,3-Dioxygenase Pathway as Immune Modulators

David H. Munn. Medical College of Georgia, Augusta, GA.

Established tumors create a state of functional unresponsiveness (tolerance) toward their own antigens that act as a fundamental impediment to cancer immunotherapy. Indoleamine 2,3-dioxygenase (IDO) is an endogenous molecular mechanism of acquired peripheral tolerance. IDO has been shown to contribute to acquired tolerance in pregnancy, mucosal tolerance, and induced tolerance to tissue transplants. In cancer, IDO can be expressed by tumor cells, but even more importantly it can also be expressed by immunoregulatory cells of the host immune system, such as plasmacytoid dendritic cells. In preclinical models, IDO expression by dendritic cells can suppress effector T-cell activation, and can drive de novo differentiation of Foxp3+ T regulatory cells (Tregs) from $CD4^+$ T cells. IDO can also directly activate preexisting mature Tregs for markedly increased suppressor activity. In addition to promoting Treg differentiation and activation, IDO and its downstream signaling pathway GCN2 kinase can act to block differentiation along the proinflammatory T_H17 pathway. IDO may thus help stabilize the suppressive Treg phenotype and suppress proinflammatory T-helper cell differentiation. Orally bioavailable small-molecule IDO-inhibitor drugs are in development, and the first of these (1-methyl-D-tryptophan, D-1 MT) is in phase 1 clinical trials. In preclinical models, 1 MT shows synergy when combined with a number of conventional chemotherapeutic agents. 1 MT also shows synergy with antitumor vaccines; and the combination of 1 MT and vaccine allows clonal expansion and effector maturation of tumor-specific T cells, even in mice with large established tumors. Vaccination and 1 MT can also drive reversal of Treg-mediated suppression, and promote reprogramming of preexisting Foxp3+ Tregs into polyfunctional T-helper cells in tumor-draining lymph nodes. Thus, IDO appears positioned at the intersection of several key regulatory pathways that contribute to tumor-induced immune suppression. Small-molecule IDO-inhibitor drugs may thus help in overcoming the state of functional immunologic tolerance toward tumors, particularly when combined with conventional cytotoxic chemotherapeutic agents and active antitumor immunization.

RIZ1 Regulates Proliferation of Monocytic Leukemia Cells in Response to Tumor Necrosis Factor-α Through Activation of P53

Abu Shadat M. Noman, Naoki Koide, Imtiaz I. E. Khuda, Jargalsaikhan Dagvadorj, Gantsetseg Tumurkhuu, Yoshikazu Naiki, Takayuki Komatsu, Tomoaki Yoshida, Takashi Yokochi. *Department of Microbiology and Immunology, Aichi Medical University, Nagakute, Japan.*

Purpose of the study: Activation of retinoblastoma protein-interacting zinc finger 1 (RIZ1) shows antiproliferative activity during cancer. But how activated RIZ1 inhibits proliferation and the signaling molecules involved in this mechanism is not clear yet. The implication of inflammatory mechanisms in regulating cancer is the current concern. Tumor necrosis factor (TNF)-α is an inflammatory cytokine regulates tumor microenvironment. This study was designed to understand the regulatory action of RIZ1 on the proliferation of monocytic leukemia cells in response to TNF-α.
Experimental procedures: Mouse and human monocytic leukemia cells, RAW 267.4 and U937, were stimulated with TNF-α at different dose-dependent and time-dependent manners and the expression of RIZ1 was analyzed. To clarify the signal molecules triggering TNF-α – induced RIZ1 expression, cells were treated with pharmacologic inhibitors or transfected with dominant negative mutants. To investigate RIZ1 and p53 interaction, cells were transfected with RIZ1-specific (400 nM) and nonspecific small interfering RNA (siRNA) and immunoblotted with anti-p53 antibody. RIZ1 was immunoprecipitated and p53 was analyzed by immunoblotting. The effect of p53 inhibitor on TNF-α – induced RIZ1 expression was also examined by immunoblotting. The effect of RIZ1 siRNA on the proliferation of TNF-α – treated cells was examined using BrdU calorimetric assay and further confirmed by the expression of PCNA. To assess the anchorage independent growth, a soft agar colony assay was performed.
Results: RIZ1 was significantly induced by TNF-α in RAW 264.7 and U937 cells in time-dependent and dose-dependent manners. TNF-α – induced RIZ1 expression was clearly suppressed by nuclear factor-κB and AKT-dominant negative plasmid and pharmacologic inhibitors. RIZ1 siRNA inhibited the activation of p53 in TNF-α – treated cells. The presence of p53 in the RIZ1 fraction precipitated by anti-RIZ1 antibody suggests the formation of RIZ1/p53 complex. Furthermore, the augmentation of RIZ1 expression by p53 inhibitor indicates negative feedback by p53. The expression of PCNA and cell growth in TNF-α – treated monocytic leukemia cells were increased in RIZ1-silenced cells. The down-regulation of RIZ1 with the siRNA enhanced the soft agar colony formation in TNF-α – treated cells.
Conclusions: RIZ1 was induced by TNF-α and the expression was dependent on nuclear factor-κB and AKT signaling. Further, silencing of RIZ1 prevented p53 activation and augmented the proliferative activity of TNF-α – treated cells. Here, we demonstrate that RIZ1 negatively regulates the proliferation of TNF-α – treated monocytic leukemia cells through activation of p53. The RIZ1-p53 interaction might explore many questions to understand tumor suppressive mechanism of p53.

The Antiproliferative and Antiviral Effect of Interferon-β on Breast Cancer Cell Line and its Mutants

Sang-Min Park, Jun-Woo Ahn, Shin Wha Lee, Dae-Yeon Kim, Jong-Hyeok Kim, Yong-Man Kim, Joo-Hyun Nam, Young-Tak Kim. *Department of Obstetrics and Gynecology, University of Ulsan, Asan Medical Center, Seoul, South Korea.*

Objectives: Recently, many researches have been conducted to develop novel strategies of individualized chemotherapy, which could overcome the cancer resistance mechanisms and toxicity of established chemotherapeutic agents. Interferon (IFN)-β stimulates both macrophages and natural killer cells to elicit an antiviral response, and is also active against tumors; however, it has not been used in patients until now because the mechanisms of resistance have not been well known. In this study, the antiproliferative and antiviral effects of IFN-β on the human breast cancer cell line (MDA-MB-468) and its mutants, and its possible mechanisms were examined.
Materials and Methods: Human breast cancer cell line (MDA-MB-468) was cultured in the media containing IFN-β to obtain mutant cell lines, which are resistant to IFN-β. The antiproliferative and

antiviral effects of IFN-β were compared between MDA-MB-468 and its mutants. The expression rate of mRNA for IFN-β was evaluated by reverse transcription-polymerase chain reaction in mutant cell lines (R2) to analyze the resistance mechanisms to IFN-β.
Results: The proliferation of MDA-MB-468 was inhibited by IFN-β and its antiproliferative effect showed a positive correlation with the dose of IFN-β; cell viability was about 20% at a maximum dose of IFN-β. The antiproliferative effect in mutant cell lines showed a positive correlation with the dose of IFN-β, especially R2 and R10, although they were decreased compared with the results in MDA-MB-468. The antiviral effect had a dose-dependent relation and MDA-MB-468 had been affected more significantly than its mutants. The expression of IFN-β gene on R2 mutants by real-time reverse transcription-polymerase chain reaction was increased by over 10-fold when compared with that of MDA-MB-486.
Conclusions: The antiproliferative and antiviral activity of IFN-β was disclosed with dose dependence on MDA-MB-468 and its mutant cell lines, which are resistant to IFN-β. The overexpression of IFN-β mRNA in a mutant suggests one of the mechanisms of IFN-β resistance. Further studies could explain the resistance to IFN-β on breast cancer.

KI-67 as a Target for Nanotechnology-mediated Light Activatable Cancer Therapy

*Ramtin Rahmanzadeh**, Prakash Rai*, Adnan Abu-Yousif*, Imran Rizvi*, Jon Celli*, Bettina Baron-Lühr†, Johannes Gerdes†, Tayyaba Hasan*. **Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA; †Department of Immunology and Cell Biology, Leibniz Research Center Borstel, Borstel, Germany*.
Objectives: Expression of the nuclear Ki-67 protein (pKi-67) is strongly associated with cell proliferation. For this reason, antibodies against this protein are widely used as prognostic tools for the assessment of cell proliferation in biopsies from cancer patients. Photodynamic therapy (PDT) is a photochemistry-based, Food and Drug Administration-approved treatment modality that has showed promising results in treating various cancers. PDT relies on the activation of certain nontoxic chemicals with an appropriate energy/wavelength of light. Here, we show a novel promising approach to target the nuclear Ki-67 protein with nanotechnology-based PDT for effective killing of proliferating cancer cells.
Materials and Methods: Anti – pKi-67 antibodies were labeled with the chromophore, fluorescein 5(6)-isothiocyanate. However, these conjugates are cell impermeants and therefore to allow for intracellular delivery, the conjugates were encapsulated inside liposomes. We then evaluated the efficacy of Ki-67 targeting using 2 in vitro ovarian cancer models—monolayer cultures and 3D cultures well established in our laboratory. Cell viability after irradiation with laser light at 488 nm was assessed using standard MTT assay for monolayer cultures whereas a Live/Dead assay kit was used for analyzing 3D cultures.
Results: After incubation of the liposomal anti-Ki-67 – FITC constructs with the ovarian cancer cell line Ovcar-5, we were able to image delivery of these constructs to the nucleoli of the cells. Irradiation of these cells led to loss of viability, which decreased to 7% after 72 hours of treatment. DAPI staining shows apoptotic bodies in treated cells suggesting apoptosis as the mechanism of cell death. Our preliminary results indicate that incubation of 3D cultures with the liposomal constructs followed by light irradiation led to the destruction of these spheres over 72 hours after treatment. Using 2 different anti – Ki-67 antibodies, where one targets the active form of Ki-67, the other one a presumably inactive form of the protein, we could show that cell killing only appears after targeting the ''active'' Ki-67 fraction.
Conclusions: We show a novel approach for targeting and elimination of proliferating cancer cells. Killing of these cells is possible with very low drug concentration of about 20 nM. Using Ki-67 as a target for treatment could be especially promising for late-stage cancers with high Ki-67 labeling index. Intriguingly, most the so-called cancer stem cells are typically positive for Ki-67. Clinical implications of these results will be discussed in the presentation.

Bortezomib Sensitizes Renal Cancer Cells to TRAIL-mediated Apoptosis In Vitro and In Vivo

*Thomas J. Sayers**, Kristen Jacobsen†, Anil Shanker*, Alan D. Brooks*. **SAIC Frederick; †CIP, NCI Frederick, Frederick, MD*.
Bortezomib (Velcade/PS-341), a specific and reversible proteasome inhibitor, sensitized a mouse renal carcinoma (Renca) to apoptosis induced by both the tumor necrosis factor family ligand, TRAIL, and an agonist antibody to the TRAIL receptor DR5. The molecular basis of this increased apoptosis seemingly involved amplification of the extrinsic cell death pathway signaling, as increased activation of caspase-8, after exposure to TRAIL, was observed in Renca cells pretreated with bortezomib. Administration of bortezomib followed by anti-DR5 to Renca-bearing mice not only reduced numbers of lung metastases, but also significantly increased long-term survival, when compared with either agent alone. Encouragingly, no evidence of toxicity was noted in the treated mice. To study the molecular mechanism in more detail, the effect of bortezomib treatment on TRAIL-mediated cell death was assessed using a panel of human renal carcinoma cell lines. Analysis of 7 human renal cell carcinomas (RCCs) showed a clear increase in the sensitivity of 4 renal lines to TRAIL after bortezomib (5 to 20 nM) treatment, whereas the remaining 3 renal lines remained resistant. This increased cell death had all the features of apoptosis. The enhanced antitumor activity of the bortezomib and TRAIL combination was confirmed in long-term tumor outgrowth assays. No difference in levels of proteasome inhibition by bortezomib in sensitized and resistant RCC was observed. No major differences were noted between sensitized and resistant cells in the levels of a variety of apoptosis proteins including cFLIPL, IAP-1, XIAP, Bcl-2, Bcl-xL, Bax, or Bid after bortezomib treatment. Furthermore, changes in expression of TRAIL receptors (DR4, DR5, DcR1, DcR2) after bortezomib treatment did not correlate well with sensitization or resistance. However, enhanced procaspase 8 activation after bortezomib pretreatment and TRAIL exposure was only observed in the sensitized RCC. This increase in procaspase 8 activation in cell extracts of sensitized RCC was observed using both enzyme assays and Western blotting for activated (cleaved) procaspase 8. In addition, immunoprecipitation of the death-inducing signaling complex showed higher levels of active caspase 8 in the death-inducing signaling complex of sensitized but not resistant RCC. These data suggest that a molecular basis for bortezomib sensitization of RCC to TRAIL might primarily involve very proximal events in the extrinsic apoptotic signaling pathway that result in an amplification of caspase 8 activity.

Funded in part by DHSS NO1-CO-12400.

Catumaxomab (Removab): The Road From Preclinical Development to Approval

Diane Seimetz. Drug Development Department, Fresenius Biotech GmbH, Munich, Germany.
Catumaxomab is a trifunctional, bispecific antibody targeting the epithelial cell-adhesion molecule (EpCAM) and CD3. It redirects T cells to EpCAM-expressing tumor cells and evokes T-cell cytotoxic responses. The Fc region of catumaxomab provides a third functional binding site, which binds and activates Fcγ receptor-positive accessory cells.
Catumaxomab has been developed as targeted therapy for the intraperitoneal treatment of malignant ascites (MA), which is a common manifestation of advanced cancers with limited treatment options. A major cause of MA is the intraperitoneal spread of tumor cells, leading to abnormal accumulation of fluid within the peritoneal cavity. EpCAM is expressed in the majority of

carcinomas spreading to the peritoneal cavity, whereas the peritoneal surface is EpCAM-negative, making it an ideal target for catumaxomab therapy. Catumaxomab has been shown to eliminate tumor cells, both in vitro and in the peritoneal cavity. By destroying tumor cells, catumaxomab interferes with the pathophysiology of MA and thereby efficiently reduces ascites formation. In April 2009, the European Commission (EC) approved catumaxomab (Removab) for the treatment of malignant ascites. It is the first drug worldwide with a regulatory label for this indication and provides an important new therapy approach. The EC therewith followed the recommendation of the Committee for Human Medicinal Products who considered by consensus that the benefit/risk balance of Removab was favorable.

The approval is based on the results of an international pivotal study (n = 258) with the primary end point of puncture-free survival, which was defined as time to the next therapeutic puncture or time to death, whichever occurred first. Treatment with catumaxomab resulted in a 4-fold increase in puncture-free survival over a therapy with puncture alone (46 d for catumaxomab vs. 11 d for control, $P < 0.0001$). Median puncture-free time was 77 versus 13 days ($P < 0.0001$). Overall survival showed a positive trend for catumaxomab versus control. The most frequent adverse events, symptoms related to cytokine release, were generally mild to moderate, and reversible.

For preclinical testing, a targeted program was designed taking into account that catumaxomab is a protein with binding properties essentially specific for humans. The program included in vitro systems and in vivo investigations with a surrogate antibody. In vitro effects of catumaxomab were assessed using human cells where the antibody is able to exert its full pharmacologic activity. A surrogate antibody (BiLu), which is of equivalent structure but binds to mouse CD3 instead of human CD3, was used for pharmacology, pharmacokinetic, and toxicology studies in mouse models.

Inhibition of Human Cervical Cancer Cell Growth by a Cyclooxygenase-2 Inhibitor, Etoricoxib

*Shweta Tripathi**, Sudhir Chandna†, Bilikere Srinivasa Dwarkanath†, Daman Saluja*, Madhu Chopra*. **Dr B. R. Ambedkar Center for Biomedical Research, University of Delhi; †Institute of Nuclear Medicine and Allied Sciences, Timarpur, DRDO, Delhi, India.*

Introduction: Cyclooxygenase (COX), also known as prostaglandin endoperoxidase or prostaglandin G/H synthase, is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins. Two forms of COX, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in many tissues, whereas COX-2 is an inducible enzyme involved in inhibition of apoptosis, potentiation of cell growth and angiogenesis, and as such is a target for drug development. Recently, clinical, cellular, and animal experimental studies have indicated its relevance to tumor invasion and metastasis. Etoricoxib is a novel nonsteroidal anti-inflammatory drug that stops the production of inflammatory prostaglandins, without stopping the production of prostaglandins that protect the stomach and intestine. The underlying signal transduction pathway is not clarified at the moment but different COX-independent mechanisms might contribute to wanted and unwanted effects of this drug. There is persuasive evidence that COX-2 inhibitors suppress cancer cell proliferation owing to their role in apoptosis. The purpose of this study was to elucidate the mechanism of action by which etoricoxib induces the proliferation arrest of HeLa cells.

Methods: Cell viability was measured by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay and Trypan blue exclusion method. Apoptosis was examined by DNA fragmentation and Comet assay and the distribution of cells in the cell cycle was estimated by flow cytometry. Western blotting was used to explore various mechanisms of etoricoxib-induced apoptosis.

Results: Drug was purified by high-performance liquid chromatography. 3-(4,5-dimethylthiazol-2yl)-2,5-Diphenyl tetrazolium bromide assay on cancerous HeLa, U-87, Hep 3B, and MCF-7 showed 60% to 70% cell toxicity. The cervical cancer cell line, HeLa, showed about 50% cell viability at a concentration of 200 μm. No cytotoxicity was seen with normal cell lines such as NIH3T3, COS 7, and HEK. Comet assay results suggest that there is DNA damage in both dose-dependent and time-dependent manners and that the precise kinetics of the progressive stages of DNA fragmentation in both the manners is attributed to apoptosis. In conclusion, our study shows that in the human cervical cancer cell line HeLa, etoricoxib displays cytostatics, induces apoptosis and causes cell death by DNA damage and thus can be used as an antiproliferative drug for cancer management.

Superior Survival of Ewing Sarcoma Family of Tumor Patients Associates With High Expression of C5 at Tumor Site

Suvi Savola*, Arto Klami†, Samuel Myllykangas*, Katia Scotlandi‡, Piero Picci‡, Cristina Manara‡, Sakari Knuutila*, *Jukka Vakkila§. *Department of Pathology, University of Helsinki; †Department of Information and Computer Science, Helsinki University of Technology, Espoo; §Hematology Research Unit, Helsinki University Central Hospital, Helsinki, Finland; ‡Laboratorio di Ricerca Oncologica, Instituti Ortopedici Rizzoli, Bologna, Italy.*

Unlike in most adult-onset cancers, an association between typical pediatric neoplasias and inflammatory triggers is rare. We studied whether immune system-related genes are activated and have prognostic significance in Ewing sarcoma family of tumors (ESFT). Data analysis was performed on gene expression profiles of 44 ESFT patient samples, 11 ESFT cell lines, and 18 normal muscle tissue samples. Two hundred thirty-eight inflammation-related genes were selected based on the literature and a macrophage gene expression signature was derived from SymAtlas. Differential expression of the genes was analyzed by *t* test and survival analysis was performed according to the gene expression. Results indicated that inflammatory genes are activated in ESFT patient samples, as 37 of 238 (16%) inflammatory genes were significantly up-regulated ($P < 0.001$) when compared with ESFT cell lines. This inflammatory gene activation was characterized by significant enrichment of macrophage-related gene expression with 52 of 299 (17%) of these genes being up-regulated ($P < 0.001$). By combining the expression data from ESFT patients, ESFT cell lines, and muscle tissue samples, we were able to identify a total of 32 presumptive inflammatory genes that characterize and distinguish tumor site in vivo from the surrounding normal tissues. Of these genes, up-regulated expression of C5 correlated with better event free ($P = 0.01$) and overall survival ($P = 0.004$) in a dose-dependent manner. In addition, high expression of JAK1 correlated with favorable overall survival ($P = 0.04$). In addition, high expression of interleukin-8, derived presumably from infiltrating immune cells, associated with poor overall survival in classical Ewing sarcoma (n = 33) ($P = 0.04$). C5 expression at protein level by ESFT cells was confirmed by immunohistochemistry. In conclusion, immune system-related gene activation was observed in ESFT patient samples and several prognostically significant inflammatory genes (C5, JAK1, and interleukin-8) that could serve as potential drug targets for ESFT were identified.

Transcriptional Alteration Induced by Treatment of Traditional Chinese Medicine (PHY-906) in Colon Cancer

*Ena Wang**, Scott Bussom†, Jinguo Chen*†, Courtney Quinn*†, Lam Wing†, Zaoli Jiang†, Dan Xi‡, Libin Jia‡, Jeffrey White‡, Francesco Marincola*, Yung-Chi Cheng†. **Department of Transfusion Medicine, NIH; †Office of Cancer Complementary and Alternative Medicine, NCI, Bethesda, MD; ‡Department of Pharmacology, Yale University School of Medicine, New Haven, CT.*

PHY906 is a novel formulated GMP grade traditional Chinese medicine currently explored as adjuvant for cancer chemotherapy. It is originated from a traditional Chinese medicine formula

Huang-Qin-Tang, which has been used in China for over 1800 years for the treatment of gastrointestinal ailments, including diarrhea, nausea, and vomiting. These symptoms are similar to the side effects of cancer chemotherapy. Prior clinic observation concluded that patients receiving PHY906 in conjunction with chemotherapy treatments not only experienced an alleviation of their gastrointestinal symptoms, but also experienced slowed tumor progression. To understand the mechanism of pharmacodynamics of PHY906, we investigated the transcriptional effects of PHY906 on colon cancer with and without chemotherapy in mice.

Methods: Whole transcriptome profiling analysis was performed on 38 samples collected from mice with established colon cancer at 72-hour time points posttreatment. The treatment groups include PHY906 only, chemotherapy (CPT-11) only, a combination of CPT-11 + PHY906, and a control (phosphate-buffered saline).

Results: Multiway analysis of variance of 4 treatment groups revealed 1348 differentially expressed genes. The effect of PHY906 on colon cancer at molecular level was identified by direct comparison with control group. On the basis of the gene functional annotation, PHY906-induced gene alteration predominate in modulating the cells' inflammatory response, including those associated with CXCL9 and CXCL11 expression, Toll receptor expression, GM-CSF signaling pathways, as well as several interferon signaling pathways. In combination with chemotherapy, PHY906 synergy chemo alone induced gene alteration in quantitative fashion.

Conclusions: Our in vivo mouse model study showed that PHY906 alone may be potent in anti-inflammatory activity. Together with chemotherapy, PHY906 facilitated chemo-induced tumor growth thwarted by altering a unique set of gene expression including inflammation-related genes products and in the meanwhile could reduce inflammation reaction as side effect resulting from the chemotherapy.

Type-1 – polarized Dendritic Cells (αDC1) Loaded With Autologous or Allogeneic Tumor Material are Effective Inducers of Tumor-specific Cytotoxic T Lymphocytes Against CLL, Prostate, Colorectal, Ovarian, and Endometrial Cancer

*Eva U. Wieckowski**†, Je-Jung Lee*†, Robbie Mailliard*†, Ravikumar Muthuswamy*†, Gurkamal Chatta†‡, Herbert Zeh*†, Kenneth Foon†‡, George L. Maxwell§, Ron Herberman†, Robert Edwards†‡, David Bartlett*†, Pawel Kalinski*†. **Departments of Surgery; ‡Medicine, University of Pittsburgh; †University of Pittsburgh Cancer Institute, Pittsburgh, PA; §Walter Reed Army Medical Center, Bethesda, MD.*

To develop effective vaccines against CLL, prostate, colorectal, endometrial, and ovarian cancers, we compared the ability of different types of dendritic cells (DCs) from cancer patients to cross-present tumor antigens from ultraviolet (UV)-sterilized autologous tumor material or from allogeneic tumor cells and to induce tumor-specific cytotoxic T lymphocytes (CTLs). Monocyte-derived DCs were matured in "standard" cytokine cocktail [standard (s)DCs; matured in interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and prostaglandin E_2) or in αDC1-polarizing cocktail (matured in IL-1β, tumor necrosis factor-α, interferon (IFN)-α, IFN-γ, and poly-I:C; αDC1s). αDC1s expressed substantially higher levels of CD86 than sDCs or immature DCs whereas the expression of CCR7 showed intermediate level of expression. αDC1s showed 18-fold to 197-fold higher production of IL-12p70 during or after maturation, compared with sDCs. As a source of tumor-associated antigens, autologous tumor tissue that was sterilized and rendered apoptotic using UVB, or UVB-killed allogeneic tumor cells, were added to DC cultures at the beginning of maturation. In all the cases, tumor cells reduced the levels of IL-12 produced during the maturation, but did not significantly affect high ability of (tumor-loaded) αDC1 to produce IL-12 after their subsequent stimulation. αDC1s loaded with autologous or allogeneic tumor cells induced much higher (6-fold to 70-fold) numbers of major histocompatibility complex class 1-restricted functional $CD8^+$ T cells against both the tumor cells (CLL, prostate, colon, ovarian, and endometrial cancer cells) and against tumor-related peptide epitopes, as measured by IFN-γ enzyme-linked immunosorbent spot and 51Cr-based CTL assays. Interestingly, the high activity of αDC1s in inducing tumor-specific CTLs was not dependent on the presence of CD40L during CTL priming, suggesting the ability of αDC1-based vaccines to function in the absence of CD4 help. Our data show that αDC1s are potent inducers of tumor-specific T cells, and that UV-sterilized autologous or allogeneic tumor cells are suitable source of tumor-relevant antigens, helping to develop improved immunotherapies for patients with CLL, prostate, colon, endometrial, and ovarian cancers, even in patients with difficult to obtain sterile tumor material and with unknown specific tumor rejection antigens.

This work partially supported by a grant from NCI, NIH, UO1-Ca-063477

Supported by the grants from NIH (1PO1CA101944 and 1POCA1132714), DOD (W81XWH-09-2-0051), and Pittsburgh Foundation.

Cross-talk Between Constitutive Antiviral State in Cancer Cell Lines and the Host's Immune Response During Oncolytic Therapy

*Andrea Worschech**†‡, Maria L. Ascierto*, Nanhai Chen†, Yong A. Yu†, Qian Zhang†, Giovanni DiPasquale‖, Zoltan Pos*, Ena Wang*, Aladar A. Szalay†§, Francesco Marincola*. **Transfusion Medicine; ‖Gene Therapy and Therapeutics Branch, National Institutes of Health, Bethesda, MD; †Research and Development, Genelux Corporation, San Diego, CA; ‡Biochemistry; §Virchow Center for Experimental Biomedicine, University of Wuerzburg, Wuerzburg, Germany.*

The immune response of the host likely limits the effects of oncolytic therapy by limiting viral replication. Recent data suggest that the immune response also promotes tumor rejection during the oncolytic process[1] following pathways similar to those observed in other immune pathologies leading to immune-mediated tissue destruction.[2,3] Paradoxically, endogenous activation of innate immunity in cancer cells hampers the activation of the immune response of the host in vivo by limiting viral replication.

Methods: To characterize the relationship between baseline immune activation of cancer cells and viral replication, we screened the NCI-60 and other cell lines for permissivity to Vaccinia virus (VACV) GLV-1h68 or Adenovirus 5 (Ad5) infection.

Results: Seventeen hours after VACV GLV-1h68 and 2 days after Ad5 infection, cell lines (n = 75) were classified according to high (> 105), intermediate (104 < Index < 105), or low (< 104) infectivity indices (percentage of green-fluorescent protein-positive cells) in the live cell population multiplied with the geometric mean of green-fluorescent protein-positive/live cells, Table 1). Although permissivity to both viruses was similar, there was poor correlation among individual cell lines suggesting that factors in addition to constitutive immune activation modulate viral replication. Transcriptional profiling showed that replication of either virus is limited in cell lines constitutively expressing interferon-stimulated genes; superimposed, other factors such as expression of viral receptors further modulate the expression of viral proteins in a virus-specific manner. Analysis of the relationship between baseline activation of intracellular innate immunity, viral replication in vitro and in vivo, and success of oncolytic therapy in preclinical models based on organism-specific platforms (human arrays to study human cancer cell lines, mouse arrays to evaluate the response of the host, and whole genome VACV arrays to study the virus) may shed important information about the role of immunity in oncolytic therapy and the mechanism(s) leading to immune-mediated, tissue-specific rejection.

TABLE 1. (Worschech)

Infectivity Index	VACV (GLV-1h68) (%)	Ad5 (%)
High	13.3	18.6
Intermediate	56	54.7
Low	30.7	26.7

Ad5 indicates Adenovirus 5; VACV, Vaccinia virus.

References:
1. Worschech A, et al. *BMC Genom*. 2009. In press.
2. Wang E, Worschech A, Marincola FM. *Trends Immunol*. 2008;29:256–262.
3. Worschech A, et al. *Cancer Immunol Immunother*. 2009;58: 1355–1362.

Signatures Associated With Rejection or Recurrence in HER-2/Neu-positive Mammary Tumors

*Andrea Worschech**†‡, Maciej Kmieciak§, Keith L. Knutson‖, Harry D. Bear§, Aladar A. Szalay‡, Ena Wang*, Francesco Marincola*, Masoud H. Manjili§ . **NIH, Bethesda, MD; †Genelux Corporation, San Diego, CA; §Virginia Commonwealth University Massey Cancer Center, Richmond, VA; ‖Mayo Clinic College of Medicine, Rochester, MN; ‡University of Wuerzburg, Wuerzburg, Germany*.

We have previously shown T-cell – mediated rejection of the neu-overexpressing mammary carcinoma cells (MMC) in wild-type FVB mice. However, after the rejection of primary tumors, a fraction of animals experience a recurrence of a neu antigen-negative variant of MMC (tumor evasion model), after a long latency period. In this study, we determined that T cells derived from wild-type FVB mice can specifically recognize MMC by secreting interferon-γ and can induce apoptosis of MMC in vitro. Neu-transgenic (FVBN202) mice develop spontaneous tumors and cannot reject it (tumor tolerance model). To dissect the mechanisms associated with the rejection or tolerance of MCC tumors, we compared transcriptional patterns within the tumor microenvironment of MMC undergoing rejection with those that resisted it either because of tumor evasion/antigen-loss recurrence (neu antigen-negative variant tumors) or because of intrinsic tolerance mechanisms displayed by the transgenic mice. Gene profiling confirmed that immune rejection is primarily mediated through the activation of interferon-stimulated genes and T-cell effector mechanisms. The tumor evasion model showed combined activation of Th1 and Th2 with a deviation toward Th2 and humoral immune responses that failed to achieve rejection likely because of the lack of target antigen. Interestingly, the tumor tolerance model instead displayed immune suppression pathways through activation of regulatory mechanisms that included in particular the overexpression of interleukin-10, interleukin-10 receptor, and suppressor of cytokine signaling (SOCS)-1 and SOCS-3. These data provide a roadmap for the identification of novel biomarkers of immune responsiveness in clinical trials.

VIRAL & CELLULAR PROTEOMIC TARGETS

Targets of Protective Tumor Immunity

Glenn Dranoff. Dana-Farber Cancer Institute, Boston, MA.

The detailed analysis of patients achieving sustained clinical benefits from irradiated, autologous granulocyte macrophage-colony stimulating factor secreting tumor cell vaccines and cytotoxic T lymphocyte antigen-4 antibody blockade affords a rich opportunity to identify antigens associated with immune-mediated tumor destruction and to delineate mechanisms of therapeutic immunity. We elucidated several of the molecular pathways that underlie this dual targeting of melanoma cells and the tumor vasculature. We established a key role for the NKG2D system, as some long-term responding patients mounted high-titer antibodies to major histocompatibility complex class 1 chain-related protein A (MICA), an NKG2D ligand, and ERp5, a protein disulfide isomerase involved in MICA shedding. The anti-MICA antibodies proved functional, antagonizing the immune suppression triggered by soluble MICA, and stimulating innate and adaptive antitumor cytotoxicity. These results have motivated several companies to develop anti-MICA monoclonal antibodies as cancer therapy. In addition, we uncovered a potent humoral reaction that broadly targeted the angiogenic network within the tumor microenvironment. Treatment-induced antibodies to angiopoietin-1/2 and macrophage inhibitory factor blocked the ability of these cytokines to promote angiogenesis in several in vitro assays. These results suggest that immunotherapies might be effectively combined with antiangiogenic strategies. We also revealed an association between coordinated humoral reactions against multiple intracellular proteins and immune-mediated tumor destruction in some patients. Lastly, we found that the immunogenicity of some target antigens is conserved between mice and humans.

Cell-free Epstein-Barr Virus DNA is a Specific Biomarker for Tumor Burden in EBV-associated Lymphomas

*Kimberley Jones**, J. Nourse*, P. Crooks*, D. Gottlieb†, Maher Gandhi*‡. **Clinical Immunohaematology Laboratory, Queensland Institute of Medical Research; ‡Department of Haematology, Princess Alexandra Hospital, Brisbane, QLD; †Department of Haematology, Westmead Hospital, Sydney, NSW, Australia*.

The quantity of Epstein-Barr virus (EBV) DNA in the peripheral blood of EBV-positive lymphoma patients has the potential to be a highly specific biomarker that would assist in the clinical management of these patients. A number of studies have shown that cell-free tumor-derived DNA is elevated in cancer patients. The presence of EBV within the malignant cells of EBV-associated cancers and the elevated level of EBV DNA in the blood of these patients makes it a highly attractive biomarker. Numerous studies have illustrated that EBV DNA is elevated in the blood of certain EBV-positive lymphoma patients and that the viral load is nil at remission but elevated in refractory disease or returns at relapse. However, there is yet to be a detailed study that incorporates serial monitoring with radiographic imaging. Furthermore, EBV viral load analysis remains a controversial topic as the optimal blood compartment to analyze and the most appropriate target gene to amplify is yet to be determined.

We report the results of a multicenter, prospective study on 40 lymphoma patients (15 female and 25 male). Serial samples were taken at 6 fixed time points during the course of therapy. The mean age was 45 years. The tumor load and response were determined by computed tomography (± positron emission tomography). Their plasma, serum, and peripheral blood mononuclear cell (PBMC) EBV viral load was determined by real-time polymerase chain reaction. To establish whether a multiple copy gene increases the sensitivity of the polymerase chain reaction, we amplified 2 genes, the single copy gene BALF5 and the multiple copy gene BAMH1W.

Of the 40 lymphoma patients, 14 had EBV-positive lymphomas and of these 14 patients, 11 had a positive EBV viral load in their plasma (positive viral load defined as above cut-off value of 100 copies/mL of plasma). Of those patients who had a detectable viral load before treatment, there was a tight correlation between the kinetics of the viral load and tumor burden, with viral load decreasing and remaining below threshold in sustained responders and remaining above threshold in patients with refractory disease. The BALF5 and BAMH1W results were analogous, indicating similar sensitivity.

In contrast to the plasma, there was no association between tumor burden and viral load measured from the patient PBMC, and no correlation between matched PBMC and plasma samples. This suggests that the cell-free compartment is the optimal one to analyze, with PBMC (and by inference whole blood) overly sensitive due to the detection of EBV in benign B cells. Our results indicate that cell-free EBV DNA is a highly sensitive and specific biomarker that reflects tumor load of patients with EBV-positive lymphomas.

Mechanisms Underlying the Selective Impairment of Epstein Barr Virus-Nuclear Antigen 1-specific Effector T Cells Observed in PTLD

*Kimberley Jones**, J. Nourse*, L. Morrison†, Frank Vari*, D. Moss†, Maher K. Gandhi*‡. **Clinical Immunohaematology Lab; †EBV Biology Lab, Queensland Institute of Medical Research; ‡Department of Haematology, Princess Alexandra Hospital, Brisbane, QLD, Australia*.

In Epstein-Barr virus (EBV)-driven PTLD, evasion was believed to be simply the result of iatrogenic immunosuppression leading to the absence of EBV-specific cellular surveillance. If this was true, EBV-specific immunity would be "globally" depressed. Conversely, if lymphoma-driven mechanisms operate this would result in selective impairment against only those EBV antigens expressed within the diseased lymph node. Although EBV-latent antigen expression in PTLD varies with the time of onset, EBV-nuclear antigen 1 (EBNA1) is universally expressed. EBNA1 may offer a viable antigenic target for the treatment of PTLD. To date, no study of EBNA1-specific T-cell immunity has been undertaken. A deeper understanding of the mechanisms used by the lymphoma cell to evade EBNA1 is needed to optimize immunotherapeutic strategies.

Cytotoxic T lymphocyte (CTL) proliferation, interferon-γ, and CD107 granule release were studied in 14 PTLD patients. Blood was taken before therapy and results compared with 19 healthy EBV-seropositive controls. T cells were expanded using 17-mer overlapping peptide pools directed against the EBV antigens EBNA1 or the lytic protein BZLF1 (the latter is not expressed in PTLD) in a 14-day culture and assayed by flow cytometry.

Strikingly, we observed a 4-fold to 5-fold reduction in both interferon-γ and CD107-releasing EBNA1-specific $CD8^+$ T cells in PTLD patients as compared with controls. By contrast, BZLF1-specific CTLs were unimpaired relative to controls. EBNA1-specific $CD4^+$ T cells were unaffected. Our data are consistent with a lymphoma-specific inhibition mediated within the tumor microenvironment.

Our group, and others, has previously described a variety of different immune evasion mechanisms associated with the impairment of tumor-associated antigen-specific immunity in "overtly immunocompetent" lymphoma patients. We hypothesized that these mechanisms might explain the EBNA1-specific inhibition observed in PTLD. PD1 was up-regulated on EBNA1-specific human leukocyte antigen class 1 pentamer-positive CTL lines and PDL1/PDL2 were elevated in 16 primary patient PTLD B-cell lines. However, real-time polymerase chain reaction revealed normal levels of PDL1/ PDL2 messenger RNA in primary patient biopsies. Similarly, messenger RNA for the immune inhibitory molecules, Galectin-1 and Galectin-9, was not elevated.

We show a selective impairment of immunity against a highly relevant tumor-associated antigen. Effective vaccine strategies will require generation of a spectrum of EBNA1-specific CTL clonotypes. T-cell receptor analysis is ongoing to establish whether EBNA1-specific CTL T-cell receptor repertoire in PTLD patients is restricted compared with healthy controls.

Current and Future Preventive Human Papillomavirus Vaccines

*Douglas R. Lowy**, Patricia M. Day*, Rhonda Kines*, Jeffrey N. Roberts*, Cynthia D. Thompson*, Susana Pang*, Christopher B. Buck*, Richard Roden†, John T. Schiller*. **Laboratory of Cellular Oncology, National Cancer Institute, CCR, Bethesda; †Pathology, Oncology, Obstetrics and Gynecology, Johns Hopkins University, Baltimore, MD.*

Cervical infection by a subset of human papillomaviruses (HPVs), especially HPV16 and HPV18, is the primary cause of virtually all cases of cervical cancer, which worldwide is the second most common cause of cancer deaths in women. HPV is also responsible for a variable proportion of several other cancers, including vulvar, vaginal, penile, anal, and oropharyngeal. Identification of HPV as the causative agent of cervical cancer has led to the development of prophylactic HPV vaccines based on the observation that the L1 main structural protein of the HPV virion can self-assemble into empty virus-like particles (VLPs), which contain the conformationally dependent neutralization epitopes of L1 and can induce high levels of neutralizing antibodies. Two pharmaceutical companies have developed commercial versions of the VLP vaccine. The one manufactured by GlaxoSmithKline is a bivalent vaccine composed of VLPs from HPV16 and HPV18, whereas Merck is a quadrivalent vaccine composed of VLPs from HPV6 and HPV11 (which together account for about 90% of genital warts) in addition to VLPs from HPV16 and HPV18. The Merck vaccine is licensed in the United States (for females 9-26 y old), the European Union, and other countries, whereas the GlaxoSmithKline vaccine is licensed in Europe and other countries, but has not yet been licensed in the United States. Clinical efficacy trials conducted by the companies have shown that, for fully vaccinated women, both vaccines induce almost complete protection against incident persistent genital infection attributable to the HPV types targeted by the vaccine and the associated lesions, including high-grade cervical dysplasia and, for the Merck vaccine, genital warts. Both vaccines also confer some degree of cross-protection against incident infection by closely related HPV types. The type-restricted nature of protection implies that nearly 30% of potentially cancer-causing infections will not be prevented by the current vaccines. Therefore, it would be beneficial to develop second generation vaccines that, ideally, could protect against a broader spectrum of serious HPV infections, were less expensive to produce and deliver, and required fewer doses. Efforts are underway to develop vaccines that meet at least some of these criteria.

To test new vaccines and other potential primary prevention approaches, and to gain insight into the initial steps of HPV infection in vivo, we have developed a tractable mouse cervicovaginal challenge model in which the female mouse genital tract can be infected with HPV pseudoviruses. The pseudoviruses are composed of authentic HPV capsids that have encapsidated a reporter plasmid that can express a reporter protein (such as luciferase or red fluorescent protein) when infection is successful. Results indicate that the intact genital mucosa is resistant to virion binding and, therefore, to infection. However, disruption of mucosal integrity, by gentle abrasion with a cytobrush or a detergent (nonoxynol-9), leads to the efficient binding of virions first to the basement membrane, rather than to cells, which is followed by infection of the keratinocytes in the genital tract and expression of the reporter protein. The presumed key role of epithelial disruption may also explain the high degree of protection induced by the vaccine, as microtrauma could provide a mechanism for the preferential exudation of systemic neutralizing antibodies at sites of potential infection.

Therapeutic Strategies for Human Papillomavirus-associated Cancers

Karl Munger. Brigham & Women's Hospital, Harvard Medical School, Boston, MA.

High-risk human papillomaviruses (HPVs) are associated with a number of cancers, most notably cervical carcinoma. Only 2 viral oncoproteins, high-risk HPVs are associated with a number of carcinomas, most notably cervical carcinoma. Only 2 viral oncoproteins, HPV E6 and E7, are consistently expressed in HPV-associated cancers. The E6 and E7 oncoproteins contribute initiation and progression of HPV-associated carcinomas and their continued expression is key to the maintenance of the transformed state. Hence, HPV E6 and E7 oncoproteins are predicted to be excellent therapeutic targets. E6 and E7 are small proteins that lack intrinsic enzymatic activities and/or specific DNA-binding properties but function by associating with and functionally reprogramming host cellular regulatory networks. Hence, E6 and E7 are considered poorly "druggable" directly by small molecule inhibitors. To overcome these limitations, we are using a combination of proteomics, genetic screens, and systems biology related approaches to identify signaling pathways that are essential in HPV oncoprotein expressing cells but are dispensable in normal cells.