International Society for Biological Therapy of Cancer 23rd Annual Meeting Abstracts

(Primary Authors are Italicized)

ADOPTIVE TRANSFER

Selective Expansion of Human T Regulatory Cell Subsets and T-cell Depletion: Role of Rapamycin (Sirolimus)

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Objective: The immunosuppressive drug rapamycin promotes the expansion of regulatory T-cell subsets, CD4+CD25high Foxp3+ and interleukin (IL)-10+ Tr1 cells. The mechanisms still remain unknown. Here, we studied expansion, survival and apoptotic pathways of human regulatory T cells (nTreg) and Tr1 cells in response to rapamycin.

Methods: CD4+CD25+ and CD4+CD25− T cells were sorted from peripheral blood mononuclear cells of normal controls (n = 15) using AutoMACS. CD4+CD25+ T cells were expanded in the presence of anti-CD3/CD28 Abs and 1000 IU/mL IL-2 for 3 weeks (nTreg). Sorted CD4+CD25− T cells were co-cultured with autologous immature dendritic cells and tumor cells of HNSCC cell line in presence of low doses of IL-2, IL-10, and IL-15 for 10 days (Tr1 cells). Rapamycin (1 nM) was added to half of the cultures. After harvest, phenotype expression of survival proteins or rapamycin-induced apoptosis (AnnexinV) was determined. Regulatory function was tested in co-cultures with autologous CFSE-labeled CD4+CD25− or CD8+CD25− T responders.

Results: Expansion of CD4+CD25high Foxp3+ nTreg cells was significantly promoted after expansion of CD4+CD25+ T cells in presence of rapamycin ($P < 0.001$) and cells showed high suppressor activity (> 82%). Furthermore, rapamycin promoted up-regulation of antiapoptotic and down-regulation of proapoptotic proteins on nTreg, opposed to inverse effect on naive T cells. In vitro induction of Tr1 cells with suppressor activity in CD4+CD25− T cells was significantly augmented in presence of rapamycin, as was level of expressions of apoptotic proteins and cytotoxins on these cells. Both nTreg and Tr1 cells were resistant to rapamycin-mediated apoptosis.

Conclusions: Rapamycin models the sensitivity of effector and suppressor T cells to apoptosis favoring the survival of Treg subsets, nTreg and Tr1 cells. Thus, this drug is applicable decedent studies of these cells and may be beneficial in immunotherapy in T-cell mediated diseases.

Targeted Elimination of Brain Tumor Stem Cells With T-cell Therapies

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Human brain tumors have been shown to consist of a subset of cells, which exhibit stem cell-like properties and can drive tumor formation. Brain tumor stem/progenitor cells (BTSCs) are a formidable cellular target for current therapeutic regimens, and have been shown to be chemoresistant and radioresistant due to the high expression of multidrug resistant pumps, antiapoptotic genes, and preferential activation of the DNA damage response pathway. We hypothesize that the glioma cancer stem cell population represents a clinical entity that is attractive for cellular immunotherapeutic intervention. However, at present little is known regarding the immunobiology of BTSCs, including their intrinsic ability to be recognized by cytolytic T lymphocytes (CTLs) based on antigen presentation and antigen processing, their sensitivity to CTL-mediated effector mechanisms such as perforin/granzyme lytic pathways, or their employment of potential escape mechanisms that render CTLs anergic or apoptotic.

We have expanded the CD133+ cancer stem cell population from primary human brain tumors and have demonstrated that these cells do exhibit stem cell-like properties: they (1) grow in neurosphere-like clusters; (2) self-renew in vitro to reform secondary neurospheres; (3) express stem cell markers; (4) differentiate to express lineage specific markers; and (5) form tumors in nonobese diabetic-severely compromised immunodeficient mice. We are currently evaluating the utility of T cells for BTSC elimination. Using target populations that are either loaded with a cytomegalovirus (CMV) pp65 immunodominant peptide or engineered to express CMV pp65, we show that BTSCs are killed by CMV-specific T cells as efficiently as matched differentiated tumor lines in vitro; and CMV-specific CTL are capable of ablating the in vivo tumor initiation of ex vivo expanded pp65+ BTSC tumor spheres. Furthermore, we demonstrate that chimeric immunoreceptor redirected IL13Rα2-specific CTL, presently being evaluated in an FDA-approved pilot phase I trial, can kill IL13Rα2-expressing BTSCs in vitro, and reduce the engrafted potential of this population in an orthotopic murine tumor model. Current models now predict that curative therapies for many cancers will require the elimination of the stem/progenitor population, and our studies lay the foundation for an immunotherapy approach to achieve this goal.

Adoptive Transfer of "Young" MART1/Melan-A CTL Generated With Artificial APC and IL-2/IL-15: Emergence and Persistence of a Memory/Effector Phenotype

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We are conducting a "first-in-human" study of adoptively transferred MART1/Melan-A–specific cytotoxic T lymphocytes (CTL) lines in metastatic melanoma. Autologous CD8+ T cells are stimulated weekly with peptide-pulsed human cell-based artificial antigen presenting cell (APC) and expanded with low-dose interleukin (IL)-2 and IL-15. After 3 weeks, polyclonal MART1 CTLs are reinfused without chemotherapy, IL-2, or vaccination. Two reinfusions are scheduled per subject where the second graft is produced from CD8+ T cells harvested 14 days after the first reinfusion. To date, $\geq 4 \times 10^9$ CTL with potent effector function and central effector memory phenotype were successfully generated for all enrolled subjects. Five CTL infusions were administered to 3 subjects at $2 \times 10^8/m^2$ (39% MART1 multimer positivity, median). Clinically, all infusions were well tolerated. Multimer staining showed that, immediately postinfusion, the percentage of CD8+ T cells specific for MART1 temporarily increased in all subjects. In subject 2, a sustained 2.2-fold increase in MART1 T cells was observed 21 and 39 days after the second Infusion. Interestingly, in this subject, immunophenotyping of peripheral MART1 CTL converted from a naive to a mixture of naive/memory phenotypes. We identified 10 individual MART1 T-cell clonotypes from peripheral CD45RA– memory T cells on day 21. Clonotypic TCR Vβ CDR3 analysis revealed that 7 out of 10 of these clonotypes existed in CTL grafts. Furthermore, 4 clonotypes persisted in the peripheral CD45RA– memory fraction on day 39. This data suggests that

adoptively transferred "young" MART1 CTL are endowed with the capacity to persist in vivo without additional immunomodulation. In subject 3, who showed a mixed clinical response, 5 individual MART1 T-cell clonotypes were isolated from lung metastases. Four out of 5 clones were included in the CTL grafts. This finding supports the possibility that infused CTL can traffic and localize to sites of disease. Intriguingly, in both subjects, we were able to identify MART1 CTL clonotypes that were not detectable in the CTL grafts but possibly emerged after CTL infusion, indicating that adoptive transfer of MART1-specific CTL may provoke a de novo antitumor response. Taken together, these results suggest that "young" MART1 CTL generated ex vivo with a cell-based artificial APC in the presence of IL-2 and IL-15 may persist in vivo and induce de novo anti-tumor responses. After completion of initial safety studies, we will introduce other immunomodulators such as lymphodepletion, IL-2 administration, and/or checkpoint blockade with anti-CTLA4 monoclonal antibody to our artificial APC-based adoptive T-cell therapy.

Insertion of an MHC Class I-restricted T-cell Receptor (TCR) Skews the Phenotype of Genetically Engineered Human Peripheral Blood Mononuclear Cells (PBMCs) for Adoptive T-cell Therapy

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Immunotherapy based on adoptive transfer of ex vivo expanded tumor infiltrating lymphocytes, and more recently, T cells engineered to express T-cell receptors (TCRs) directed to tumor antigens, have been shown to be effective as a treatment for metastatic melanoma. We set up to define the effects on cell phenotype and function of the insertion of a foreign TCR into ex vivo expanded peripheral blood mononuclear cells (PBMCs).

We stimulated human PBMCs from different donors with anti-CD3 antibody (OKT3) and interleukin-2. On days 2 and 3, cells were transduced in retronectin-coated plates with retrovirus (RV) expressing a high affinity/avidity Melan-A/MART-1 TCR (kind gift from R. Morgan and S.A. Rosenberg, NCI) or a control fluorescent, green fluorescent protein (GFP). Transduction efficiency was very high, routinely $>80\%$ when assessed by GFP and $>70\%$ for surface MART-1 TCR expression by tetramer assay.

Immunophenotyping for CD4 and CD8 T-cell subsets in MART-1 TCR expressing PBMCs demonstrated a skewing toward the CD8 phenotype that was TCR-dependent but not transduction-dependent. RV-GFP–transduced cells maintained the normal ratio of CD4+ to CD8+ T cells throughout the study period. However, RV–MART-1 TCR-transduced cells demonstrated a progressive reversal of the CD4/CD8 throughout the 25-day ex vivo expansion period, with 17% to 48% of CD4+ T cells and 51% to 83% of CD8+ T cells in replicate experiments at culture day 16. Absolute number counting and CFSE labeling studies indicated slower proliferation of CD4+ T cells in contrast to high CD8+ T-cell expansion upon MART-1 TCR engineering and not with GFP control.

Functional assays to detect specific interferon-γ (IFN-γ) production demonstrated exquisite specificity to MART-1 by the TCR-engineered cells regardless of the timing of analysis. We determined IFN-γ production by enzyme-linked immunosorbent assay, cytometric bead array, and intracellular staining in ex vivo expanded PBMCs at 7, 10, 15, and 35 days after MART-1 TCR transduction. There was high level of antigen-specific IFN-γ secretion ($>11\times10^3$ pg/mL per million cells/24 h) specific for MART-1 peptide pulsed K562/A2.1 or dendritic cells, and HLA-A2.1+/MART-1+ melanoma cell lines, Similarly, cytotoxic T-cell activity by Cr51 release assays indicated specific lysis of HLA-matched melanoma cell lines.

In conclusion, insertion of a major histocompatibility complex class I-restricted TCR skews the phenotype of PBMC to favor CD8+ CTL development with high antigen-specific functional activity, making them a good source for TCR-redirected T cells for an upcoming TCR-engineered clinical trial.

Intralesional Placement of Lymphokine-activated Killer (LAK) Cells After Resection of Primary Glioblastoma (GBM)

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Background: Median survival for resectable glioblastoma (GBM) patients is only 12 to 15 months, even with the addition of intraoperative BCNU chemotherapy wafers or adjuvant temozolomide; so an additional effective adjuvant treatment would be desirable. We previously observed minimal toxicity and an encouraging 9-month median survival and 34% 1-year survival from the date of treatment with intralesional autologous lymphokine-activated killer (LAK) cells in 40 patients with recurrent GBM (*J Immunother*. 2004;27:398–404,). The purpose of the current study was to obtain safety and efficacy data for the use of LAK cells placed intralesionally in patients with surgically proven GBM as part of primary therapy rather than after disease progression.

Methods: Eligible patients had completed primary therapy for GBM per their managing physician without disease progression. LAK cells were produced by incubating peripheral blood mononuclear cells after Ficoll-hypaque separation with 6000 IU/mL interleukin-2 in AIM-V media in culture bags at a cell concentration of 3×10^6/mL for 3 to 5 days. The harvested LAK cells were then suspended in autologous plasma with 1 MIU interleukin-2, to which calcium was added to produce a fibrin clot. The LAK preparation was then transported to the operating room where the surgeon placed the cells into the surgically exposed tumor cavity.

Results: LAK cell production was satisfactory for all 36 patients, including 22 men and 14 women aged 35 to 78 years with a median age of 57. All but 1 had undergone prior neurosurgery (18 had near complete resection; 13 had a partial resection). All patients had received partial brain radiation and a gamma knife boost except for 1 patient who had only undergone a near complete resection and gamma knife therapy and another who had only received irradiation via gamma knife. Twenty-four had received chemotherapy (92% temozolomide) prior to LAK. LAK treatment was well tolerated. Average length of hospitalization was 3 days and median 2 days. Median time from diagnosis to LAK cell therapy was 5.0 months (range, 3 to 11). At the time of this analysis, 25 patients have died, but the median survival from the date of original diagnosis is 22.5 months with a 1-year survival rate of 79%. From the time of LAK cell placement, 1-year survival is 67% with a median survival of 14.6 months.

Conclusions: This treatment is feasible, safe, and the survival encouraging. Our intent is to conduct a randomized phase II trial of intralesional therapies with LAK in 1 arm and BCNU wafers in the other arm.

Engineering GVL by T-cell Genetic Modification

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Disease relapse is a major contributor to treatment failure of hematopoietic stem cell transplantation (HSCT) for hematologic malignancy. Targeting posttransplant minimal residual disease with antigen-specific immunologic effector cells is a conceptually attractive strategy to consolidate the antitumor effect of the transplant preparative regimen by the selective augmentation of the graft-versus-leukemia (GVL) effect in the allogeneic setting. Endowing T cells with tumor specificity by genetic modification is one approach to generating effector cells for posttransplant cellular immunotherapy. To target malignant B cells of lymphoma and leukemia, we have constructed chimeric immunoreceptors specific for B-cell lineage markers by fusing CD20-specific and CD19-specific single chain antibody (scFvFc) domains to the intracellular sequence of the T-cell receptor complex's zeta chain (scFvFc:zeta). These antibody-based chimeric receptors bind to epitopes on native cell surface CD20 and CD19 and thus are non-MHC restricted and universal. Our laboratory has focused on studying the immunobiology of T cells engineered to express these receptors, as well as, on the development of these technologies for clinical deployment.

Our initial clinical trials applying autologous CD20-specific CD8+ cytotoxic T lymphocyte clone adoptive transfer for intermediate grade CD20+ diffuse large cell lymphomas and CD19-specific polyclonal T-cell autografts for follicular lymphoma have revealed a significant

obstacle to therapeutic efficacy: that being limited in vivo persistence. In order to address this, our group in collaboration with Dr Stanley Riddell's laboratory have sought to identify T-cell subsets that have the intrinsic capacity to persist following adoptive transfer and to couple the use of these cells with ex vivo culture systems for human T-cell gene transfer and expansion that preserve this function. To this end, we have identified antiviral memory T cells (Tcm's) as intrinsically programmed for in vivo persistence. We have developed an ex vivo platform system for rapid acquisition of cytomegalovirus-specific Tcm's through CD62L selection followed by cytomegalovirus pp65 activation/interferon-γ capture/self-inactivating lentiviral transduction and expansion in interleukin-15 that permits the isolation of therapeutically relevant numbers of bispecific pp65xCD19 Tcm's in 21 days.

Our group is in the final stages of manufacturing and release testing the clinical-grade reagents to make this platform operational in IND-supported clinical applications. A major new application of this technology will be toward the augmentation of GVL effect following allogeneic HSCT for CD19+ acute lymphoblastic leukemias and to explore the therapeutic application of autologous HSCT in combination with adoptive transfer of CD19-specific effectors for engineered autologous GVL for those patients without donors or who have contraindications for allografting.

Mycophenolate Mofetil Selection of Gene Modified T Cells With an Engineered Human Inosine Monophosphate Dehydrogenase II (IMPDH2)

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Endowing T cells with resistance to lymphotoxic drugs by their genetic modification affords the opportunity for in vitro and in vivo selection. The clinical use of bacterial drug resistance genes such as neomycin and hygromycin phosphotransferases is problematic due to the immunogenicity of these transgenes, as well as the incompatibility of G418 and hygromycin for direct administration to humans for in vivo selection. Thus, we are interested in examining alternative transgenes for conferring in vivo resistance of human T cells to drugs that are compatible with human use. Here, we report on the use of an inosine monophosphate dehydrogenase II double mutant (IMPDH2dm; Thr333→Ile333, and Ser351→Tyr351) transgene that confers resistance of cells to the clinically relevant immunosuppressive drug mycophenolate mofetil (MMF, a prodrug of mycophenolic acid, MPA). Studies using Daudi and Sup-B15 human B-lineage tumor cells, OKT3-stimulated peripheral blood mononuclear cell derived human T cells and established human T-cell lines demonstrated their sensitivity to MMF at 0.5 μM or more. Using CD19t-IMPDH2dm and GFP-IMPDH2dm lentiviral constructs, we were able to derive CD19t+ and GFP+ stable integrants in the presence of 1 to 3 μM MPA. These data demonstrate the utility of this human enzyme transgene selection system for genetic modification of human T cells. Using mouse xenograft model systems, current studies are evaluating the in vivo selection efficiency of IMPDH2dm expressing T cells. Future work will evaluate the ability of this selection transgene to enforce the expression of a second therapeutic transgene (ie, the chimeric antigen receptor CD19R which targets CD19+ B-lineage malignancies) and drive the selected T-cell mediated antitumor responses.

Rapid Expansion of Melanoma TIL in Adoptive Cell Therapy Leads to Loss of CD28 and Reduced Proliferative Potential in the MART-1–specific T-cell Population

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Adoptive T-cell therapy (ACT) of expanded tumor-infiltrating lymphocytes (TILs) has shown great promise in the treatment of metastatic melanoma. However, a critical problem in ACT is a lack of long-term TIL persistence in many patients required for durable clinical responses. The maintenance of an effector-memory phenotype characterized by the expression of key costimulatory molecules, especially CD28 and CD27, is associated with long-term persistence of transferred TIL. In this project, we have tracked the phenotypic and functional changes in CD8+ TIL, and their tumor-antigen–specific proliferation, after long-term culture in interleukin (IL)-2. Isolated TILs were initially expanded with IL-2 from tumor fragments and then subjected to rapid expansion protocol (REP), which is the current protocol used to generate the large numbers of cells for ACT. We found that melanoma antigen-reactive TILs (MART-1–reactive) lose their capacity to proliferate after the REP when restimulated with mature dendritic cells pulsed with MART-1 peptide. In contrast, MART-1–specific TIL before REP (pre-REP TIL) proliferated well. Pre-REP TIL continued to expand with IL-2 for a minimum of a month after antigenic restimulation, while no similar expansion of post-REP TIL was found. However, analysis of CTL function by interferon-γ staining and killing assays showed that post-REP TILs were superior effector cells. Staining for both TILs revealed that CD28 expression was significantly down-regulated during the REP, while no significant decrease in CD27 occurred. TILs were sorted based on CD27 and CD28, and restimulated. Both CD27+ and CD27− TILs expanded equally well over a 7-day period when restimulated. However, restimulated sorted CD27– TIL exhibited greater rates of MART-1–specific T-cell loss after the initial 7-day period. When sorted CD28+ and CD28− were compared, only CD28+ TIL could be induced to divide, with CD28– TIL failed to enter cell cycle and had increased apoptosis. These results indicate that loss of CD28, and not CD27, occurs during the REP and that the absence of CD28 costimulation leads to a loss of short-term proliferative potential. In contrast, CD27 costimulation seems to be required only for the longer-term survival of expanded antigen-specific CD8+ clones. Our results also help explain why the persistence of TIL expressing both CD27 and CD28 is associated with long-term complete responses in ACT patients. Highly differentiated CD28− TIL may only mediate short-term tumor eradication and cannot expand and persist for long periods of time in vivo to mediate long-term durable clinical responses. This may explain why the majority of ACT patients receiving TIL therapy exhibit only partial and not complete clinical responses.

Maintenance of Tumor Antigen-specific Cytolytic T Cells During Expansion of TIL for Adoptive Immunotherapy

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Adoptive cell transfer (ACT) of autologous tumor-reactive T lymphocytes into patients can mediate the regression of metastatic melanoma, but this therapy requires the isolation of tumor-infiltrating lymphocytes (TILs) and their expansion in vitro to large numbers. The traditional rapid expansion protocol (REP) expands TIL approximately 3 logs in 14 days through stimulation with OKT3, irradiated allogeneic feeder peripheral blood mononuclear cells, and interleukin-2. Unfortunately, this method frequently favors CD4+ over CD8+ T-cell proliferation, and also often results in poor maintenance of tumor specificity. This therapeutic limitation led us to investigate strategies to improve T-cell expansion for ACT. In this study, we assessed whether the addition of transforming growth factor (TGF)-β could improve the REP method. Thus, TIL obtained from isolated melanoma surgical specimens were rapidly expanded in the presence or absence of TGF-β, added once on Day 0 of the REP culture. Expanded TILs were then assessed to compare proliferation, survival, phenotype, and melanoma-specific cytotoxic T lymphocyte activity and cytokine release.

Surprisingly, the addition of TGF-β at the onset of the REP dramatically improved the quality of the expanded T cells. As compared with the conventional REP method, the addition of TGF-β maintained CD8+ T-cell percentages and tumor antigen specificity, while simultaneously inhibiting the expansion of CD4+ T cells. TGF-β in this setting did not negatively affect T-cell function, as interferon-γ production and the cytolytic capacity of TIL were shown to be comparable at the per cell level. However, in bulk TIL cultures rapidly expanded with TGF-β, levels of interferon-γ production and tumor-specific lysis were much higher compared to TILs expanded using the conventional REP.

Although the mechanism(s) of action remain(s) to be fully elucidated, TCR spectratyping analysis suggested that TGF-β–expanded CD8+ TIL maintained a higher level of T-cell receptor Vβ diversity following expansion. Furthermore, TGF-β inhibited activation-induced cell death of T cells. TGF-β appeared to act not only through direct effects on TIL, but also through conditioning of the irradiated feeder cells in the REP culture. Thus, many factors likely contribute to the maintenance of CD8+ TIL and antigen specificity following T-cell expansion in TGF-β. Regardless of the mechanism of action, our results suggest that this method of TIL expansion will yield higher quality, more potent tumor-specific TIL for ACT therapy.

Engineering Tunable Homeostatic Signaling Receptors Based on IL-7R for Regulation of Proliferation, Survival, and Differentiation Status of CD8+ Cytolytic T Cells

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Adoptive cell transfer therapy is a promising immunotherapeutic approach with the potential to target both infectious and malignant diseases. The largest hurdles to overcome include selecting target antigens, improving in vitro expansion of cytotoxic T lymphocytes (CTLs), and maintaining effector function and long-term survival of CTLs postinfusion. Due to the inability of adoptively transferred CTLs to persist long term in vivo, there is a need for the development of a mechanism to provide CTLs with the appropriate signals for long-term survival and persistence. Interleukin (IL)-2, a γc cytokine, has been traditionally examined to augment the response of transferred CTLs. There are detrimental effects associated with IL-2, including activation-induced cell death and regulatory T-cell generation. In contrast, another γc cytokine, IL-7, has a nonredundant role in the support and expansion of naive cells, the conversion of effector memory cells, and homeostatic proliferation. We have demonstrated that forced expression of IL-7 receptor (IL-7R) in primary T cells using a lentiviral platform, can support the proliferation and survival of primary T cells in the presence of IL-7. A proliferative advantage was observed when culturing primary T cells with forced IL-7R in the combination of IL-2 and IL-7. We are currently investigating the synergistic signaling mechanisms underlying these effects on proliferation. Future experiments will use recombinant IL-7Rs designed to constitutively signal through a tethered IL-7 molecule, which are controlled for duration and potency via inclusion of a tamoxifen-inducible estrogen-binding domain. These studies address a major hurdle in adoptive cell transfer, which is the need to provide survival and proliferation signals in vivo postinfusion in order to sustain the desired immune response.

Genetic Approaches for Combinatorial Resistance to PD-1 and TGF-β Mediated T-cell Dysfunction in the Tumor Microenvironment

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Adoptive immunotherapy is a promising therapeutic approach for the treatment of malignancies. However, there are limitations that must be addressed to enhance therapeutic efficacy including host-employed mechanisms for tumor immune evasion. Two detrimental tumor evasion mechanisms are receptor-mediated evasion via programmed cell death-1 (PD-1, expressed on T cell) interaction with programmed cell death ligand 1 (PD-L1, expressed on tumor), and cytokine-mediated evasion via tumor secreted transforming growth factor-β (TGF-β). Both PD-1/PD-L1 and TGF-β mediated mechanisms have independently been shown to result in T-cell dysfunction, including decreased proliferation and cytotoxic function leading to decreased success in tumor therapy. Although previous research has shown that independent suppression of PD-1 or TGF-β results in the abrogation of T-cell dysfunction, ultimately leading to increased tumor killing, we are interested in determining whether these 2 mechanisms of immune evasion will cooperate additively or synergistically when down-regulated in combination. To test this, we have developed both PD-1 targeted shRNA as well as dominant negative PD-1 strategies to promote resistance to PD-1 mediated tumor immune evasion. Resistance to TGF-β has been generated through the development of a dominant negative TGF-βRII. Current studies are investigating the ramifications of resistance to these tumor immune evasion mechanisms in terms of downstream signaling targets as well as developing systems for the assessment of additive or synergistic effects in cooperative resistance. This work will address a major limitation to adoptive immunotherapy, which is the need for maintenance of T-cell effector function within the tumor microenvironment.

Novel Methods for the Generation and Characterization of Melanoma-specific T Cells From Tumor-infiltrating Lymphocytes

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The generation of T cells with specific reactivity against tumor-associated antigens is a prerequisite for effective adoptive immunotherapy. Melanoma-specific lymphocyte cultures can be established from tumor-infiltrating lymphocytes (TILs) by in vitro culture with interleukin-2. However, large numbers of tumor-specific T cells can only be generated in about 50% of the patients. We hypothesize that myeloid-derived suppressor cells (MDSCs) present in the tumor limit the activation and expansion of tumor-specific T cells. Since arginase and NOS are 2 suppressive mediators employed by MDSC, we initiated TIL cultures with an arginase inhibitor (nor-noha) and a NOS inhibitor (LNMMA). We also considered that adding anti-OX40, a costimulatory agonist of CD4 and CD8 T cells, might overcome MDSC-mediated suppression. Finally, we considered that altering the culture conditions could alter the frequency and/or effector/memory phenotype of tumor-specific T cells. To date, TIL cultures have been initiated on 9 different melanoma tumor digests. Three have been analyzed completely for frequency of tumor-specific IFN-γ secreting T-cell cloids generated from TIL. While numbers are small, it appears that different tumor preparations respond differently to the specified culture conditions. In 1 case, the addition of nor-noha and LNMMA increased the frequency of cloids containing tumor-specific T cells from 50% to 100%. In another case, anti-OX40 treatment substantially increased recovery of tumor-specific T cells. However, an 8-color analysis of tumor-specific T cells, using autologous tumor-stimulated CD107a expression as a marker of specificity, suggests that regardless of culture conditions the majority of tumor-specific (CD107a+) T cells exhibit a CCR7−/CD45RA−/CD27−/CD28−/CD57−/CD8+ early effector phenotype. These preliminary data suggest that modifying TIL culture conditions to interfere with MDSC-mediated suppression can improve the recovery of tumor-specific T cells from some tumor preparations.

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Adoptive T-cell Therapy at the MD Anderson Cancer Center: Early Results and a Promising Future

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Adoptive T-cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) has shown great promise as a method of immunotherapy against metastatic melanoma mainly through clinical trials at the NCI (Bethesda, MD). However, despite the high clinical response rates, ACT has not been widely adopted because it is believed that TIL culture and expansion methods cannot be readily standardized. Thus, evidence of successful ACT at other institutes across the United States is urgently needed to prove its efficacy and develop standardized protocols that can be universally adopted by all cell therapy facilities. Recently, we initiated

an ACT program at the M.D. Anderson Cancer Center aimed at reproducing the "NCI experience" and developing synergistic therapies boosting TIL function postinfusion. Essentially, we have followed a similar method used at the NCI, with a 5-week TIL culture from tumor fragments with interleukin-2 followed by a 2-week rapid expansion protocol (REP) using anti-CD3 activation with allogeneic irradiated feeder cells and interleukin-2. TILs were tested for antitumor reactivity using autologous or HLA class I-matched allogeneic tumor cell lines. We have accrued 130 patients so far and have successfully expanded pre-REP TIL ($> 40 \times 10^6$ cells) eligible for the large-scale REP and infusion from 70/130 patients (53%). Currently, we have treated 3 patients (all receiving a prior lymphodepleting regimen at day 7), with two-third of these patients exhibiting ongoing clinical responses in peritoneal, lymph node, and subcutaneous masses (as of July 15, 2008). We have also performed detailed tracking of TIL expansion from isolated tumor fragments in relation to the type of prior therapy, site of disease, tumor mass, patient age, and HLA class I status. No affect of patient age or HLA class I status was found on TIL expansion rates. TIL yields were also not affected by tumor size, with many small tumors (< 1 g) yielding the highest numbers of TIL. However, a significant effect of prior therapy (in the last 2 to 3 mo) was found. TILs from patients who had chemotherapy expanded relatively poorly, while those from patients who had received a prior immunotherapy, or no therapy, within the last 2 to 3 months, grew markedly better. Interestingly, we also found that prior therapy with tyrosine kinase inhibitors may negatively affect TIL yield. Thus, patient selection based on the type of prior therapy is a key issue affecting the quantity and quality of TIL for therapy. In summary, our experience so far shows that with a committed institutional infrastructure, a coordinated team effort, and a set of standardized culture methods, the current NCI ACT methodology with lymphodepletion can be successfully performed by most major cancer centers.

Three Ways to Enhance the Destructive Power of Tumor-specific T Cells

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Our goal is to design new immunotherapies for patients with advanced cancer using an iterative process of mouse and human studies. In studies performed at the NCI, Bethesda, we have observed that a variety of immunotherapies can induce objective response rates (ORR) in patients with metastatic melanoma. Cancer vaccines can induce ORR ranging from 3% to 7%, while ORR using interleukin (IL)-2 or anti-CTLA-4 range from 13% to 17%. ORR using adoptive cell therapy (ACT) plus IL-2 is higher at 34%, and this is in the absence of a lymphodepleting preparative regimen. However, 3 maneuvers might improve the function of adoptively transferred T cells.

1. Adding lymphodepleting chemotherapy prior to ACT improved ORR to 49% and adding radiotherapy has improved ORR to 72% (JCO, In press): Some of these responses are complete and long lasting. We have learned that total body irradiation augments the function of adoptively transferred cells by depleting regulatory T cells, by removing immune cells that act as "sinks" for homeostatic cytokines, and by the activating the innate immune system.
2. The use of "young" T cells with stem cell-like properties in ACT enhances their effectiveness: Much progress has been made in identifying the phenotypic and functional characteristics of cells that are associated with successful ACT of large, established tumors in mice and in humans. We have found that the acquisition of effector function of CD8+ cells is associated with senescence and can limit antitumor efficacy upon adoptive transfer. Conversely, we have described that developmental arrest of CD8+ cells can be achieved using IL-21 and that this is associated with the enhanced function of anti-tumor T cell. Unpublished findings from the laboratory indicate that the developmental arrest of CD8+ T cells can be achieved at an even earlier stage, that of the "T memory stem cell" by pharmacologically simulating Wnt signaling.
3. CD4+ T cells can be powerful antitumor effector cells. "Polarization," rather than maturation, may be a major determinant of antitumor efficacy of CD4+ T cells. Skewing CD4+ T cells toward a "Th17" phenotype has recently been found to be highly effective in the treatment of large established tumors. Efforts to translate the use of "younger" cells are currently underway in the clinic, while work with CD4+ T-cell polarization remains at an early stage of preclinical development. In conclusion, ACT represents the most effective immunotherapy for patients with metastatic melanoma and patients with bulky metastatic disease achieve an objective response. Lessons learned from this work on the use of lymphodepleting preparative regimens and an understanding of T-cell differentiation are being applied to genetically engineered T cells. ACT using with peripheral lymphocytes genetically engineered to express antitumor T-cell receptors hold promise for extending ACT therapy to patients with common epithelial cancers.

Additional Reading:

1. Description of the pmel-1 (CD8+) TCR transgenic T cell model: WW Overwijk, et al. *J Exp Med*. 2003;198:569.
2. How T regulatory and T helper cells influence tumor immunity: PA Antony, et al. *J Immunol*. 2005;174:2591.
3. An up-to-date description of the basic science of ACT: Gattinoni L, et al. *Nat Rev Immunol*. 2006;6:383.
4. Activating innate immunity: CM Paulos, et al. *J Clin Invest*. 2007;117:2197.
5. How IL-21 halts the differentiation of CD8+ T cells: CS Hinrichs, et al. *Blood*. 2008;111:5326–5333.
6. Th17-polarized CD4+ T cells in a new TCR transgenic mouse model (called TRP-1): P Muranski, et al. *Blood*. 2008;112:362.
7. The challenge of targeting tumor-associated antigens: DC Palmer, et al. *Proc Natl Acad Sci USA*. 2008;105:8061–8066.

Provision of CD4+ T Cell Help Prevents Tolerization of Tumor-specific CTLs and Enhances Tumor Immunity in a Murine Model of Prostate Cancer

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In this study, we investigated T-cell tolerance to tumor antigens using the TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model in combination with adoptive transfer of T-cell receptor (TcR) transgenic T cells with specificity for a TRAMP tumor antigen. We previously reported that adoptive transfer of CD8+ (TcR-I) cells into TRAMP mice resulted in rapid tolerization of the cells. The objective of the current study was to test the ability of CD4+ helper T cells to enhance antitumor immunity by preventing or reversing TcR-I cell tolerance. Naive tumor-specific CD4+ (TcR-II) T cells adoptively transferred into TRAMP mice became activated in lymph node, trafficked to the prostate, and initially functioned as T helper-1 cells, as measured by their ability to proliferate and secrete interleukin-2 and interferon-γ in response to their cognate tumor antigen. However, by 10 days after transfer, the TcR-II cells became tolerant of tumor antigen. We next tested whether this transient activation of TcR-II cells was sufficient to prevent TcR-I cell tolerization. Co-transfer of naive TcR-II and TcR-I cells initially enhanced the frequency, activation, survival and function of TcR-I cells and increased expression of co-stimulatory molecules on dendritic cells in the tumor-draining lymph nodes and tumor, improving their ability to stimulate naive T-cell proliferation. While a single co-transfer of TCR-II cells only delayed tolerization of TcR-I cells, we have observed that repeated transfer of TcR-II cells prevented tolerization of TcR-I cells and ultimately slowed tumor progression. These data demonstrate that while tumor-specific cytotoxic T lymphocyte may be primed in the absence of CD4 help, maintenance antitumor cytotoxic T lymphocyte activity is profoundly enhanced by the sustained provision of activated CD4+ T cells. Our current studies are aimed at understanding how provision of CD4 help reverses the immunosuppressive tumor microenvironment to assist in the design of more effective immunotherapeutic approaches for treating cancer.

Phase II Trial of Combination Therapy of Tumor Lysate-pulsed Dendritic Cells and Adoptive Transfer of Anti-CD3 Activated T Cells (ATVAC) to Lower Postsurgical Recurrence Rates of Cholangiocellular Carcinoma (CCC)

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Background: Postsurgical recurrence of cholangiocellular carcinoma (CCC) is frequent and fatal. Patients with CCC who underwent curative resection revealed the median overall survival (OS) of 21 months [95% confidence interval (CI), 11.6-30.4]. This phase II study investigated the efficacy and safety of adoptive transfer of anti-CD3 activated T cells (ATVAC) in patients who underwent curative resection for CCC to lower postsurgical recurrence rates.

Methods: Patients with CCC who underwent curative resection received ATVAC. ATVAC consisted of dendritic cells with autologous lymphocytes activated with interleukin-2 and antibody to CD3 at 7-day or 14-day intervals for 3 months. The immune responses to tumor antigens (Tgs) were monitored by delayed-type hypersensitivity (DTH). The primary end point was relapse-free survival. Secondary end points were OS, the monitoring of immune responses to Tgs, and toxicity.

Results: Between September 2000 and August 2005, 38 patients were included, with a median age of 63.2 years (39 to 81), a median tumor size of 5.7 cm. Thirty-eight patients (100%) underwent segmentectomy. On the pathologic findings, vascular invasions, intrahepatic metastases, and lymph node involvements were 81.1%, 29%, and 55.3%, respectively. The median relapse-free survival was 14 months (95% CI, 3-25). The median OS was 26 months (95% CI, 6-46). 20 patients showed positive DTH responses to Tgs. Among these patients, the median OS was 72 months. In contrast, in the group of patients with a negative DTH, the median OS was 13 months (95% CI, 9-17).

Conclusions: This result suggests that ATVAC is very effective in patients with positive DTH responses to Tgs who underwent curative resection for CCC to lower postsurgical recurrence rates.

A Phase I/II Trial of Combination Therapy of Tumor Lysate-pulsed Dendritic Cells and Adoptive Transfer of Anti-CD3 Activated T Cells (ATVAC) in Patients With Advanced Gastrointestinal (GI) Cancers

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We have previously shown that tumor lysate-pulsed dendritic cell vaccination (TP-DC) elicited therapeutic rejection of established tumor in animal models. In addition, combination of TP-DC with administration of activated T cells elicited more significant therapeutic rejection of established tumor than the single therapy alone in animal models. We, therefore sought to examine the therapeutic potency of TP-DC with administration of anti-CD3 activated T cells (CAT) against gastrointestinal cancers: 3 patients were diagnosed with pancreatic cancer, 7 patients with HCC, 3 patients with intrahepatic cholangiocarcinoma, 2 patients with esophageal sarcoma, 12 patients with colorectal cancer. Monocyte-derived DCs were generated in serum-free medium containing granulocyte macrophage-colony stimulating factor (100 ng/mL) and interleukin-4 (50 ng/mL). Patients received TP-DC intradermally 4 times every 3 weeks with CAT activated with interleukin-2 and antibody to CD3: 4 patients received 1×10^6 DC, 7 received 2×10^7, 16 received 1×10^8. The safety as well as the biologic and the clinical effects of adoptive transfer of anti-CD3 activated T cells (ATVAC) were studied. Twenty-seven patients were included. Ten of these patients had evaluable unresected tumor resistant to conventional chemotherapy. ATVAC was well tolerated; the major adverse events being fever and skin erythema of low grade (grade 1 and grade 2). No grade 3 and 4 adverse events were noted. None of the 10 patients achieved partial remission; 4 patients achieved stable disease. Thirteen of the 16 patients receiving 1×10^8 DC developed a strong positive skin reaction to tumor lysates. In 6 patients, positive skin reaction to tumor lysates was shown to be maintained in 6 months after the development of the positive skin reaction to tumor lysates. The correlation of DTH responses to tumor lysates in vivo with immune responses to lysates in vitro and clinical responses was evaluated. Patients with a positive DTH to tumor lysates revealed the increase in the number of peripheral blood lymphocytes (PBL) compared to those with a negative DTH. The increase in the number of the T cells in PBL was observed in patients with a positive DTH. Furthermore, patients with a positive DTH revealed the significant interferon-γ production by T cells stimulated with TP-DC. The duration of the positive DTH was within 6 months after the final treatment correlated with the decrease in the number of PBL in vivo and the decrease of the production of interferon-γ in vitro by T cells. The results of this phase I trial appear promising for further development in patients with advanced gastrointestinal cancers.

Intratumoral Injection of Immature Dendritic Cells (DCI) Against Gastrointestinal (GI) Cancers: A Phase I Study in Patients With Metastatic GI Cancers

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We have previously shown that direct injection of immature dendritic cells (DCI) into tumor nodules could result in significant remission of established subcutaneous tumor. We therefore sought to determine DCI for cancer treatment. Monocyte-derived DCs were generated in serum-free medium containing granulocyte macrophage-colony stimulating factor (100 ng/mL) and interleukin-4 (50 ng/mL). Immature, unpulsed DCs were resuspended in 1 mL of saline. Patients received DCI into tumor nodules 4 times, 14 days apart. In some cases, patients underwent systemic administration of interleukin 2 intravenously at 350,000 IU/body daily for 4 days consecutively after each DCI. The safety as well as the biological and the clinical effects of DCI were evaluated in a phase I clinical trial in patients with metastatic gastrointestinal cancers. Sixteen patients have been included, which had evaluable metastatic or unresected tumor resistant to the conventional chemotherapy/radiotherapy. DCI was well tolerated, the major adverse events being fever were low grade (grade 1 and grade 2) with no grade 3 and 4 adverse events. The number of DCs administered ranged from 1 to 20×10^7 cells originally generated from 4 cycles of leukapheresis products. One of the 16 patients achieved partial remission (6 mo), 12 achieved stable disease. All of these 13 patients developed strong positive skin reaction to keyhole limpet hemocyanin. Considering the late stage and progression of gastrointestinal cancers, the results of this phase I trial are encouraging and promising clinical benefit in patients with chemotherapy/radiation-resistant tumors.

The CD19 Chimeric Antigen Receptor Re-directs CMV Specific T Cells Derived From Central Memory T Cells (Bi-specific T Cells) Against Human Acute Lymphoid Leukemia (ALL)

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One strategy to enhance the efficacy of antitumor adoptive T-cell therapy is to generate bi-specific T cells, which co-express viral-specific and tumor-specific antigen receptors. The hypothesis is that these bi-specific T cells will have enhanced antitumor function in a patient through the coordinate stimulation by viral antigens that are presented in the context of endogenous professional antigen presenting cells. Central memory T cells (Tcm) have a unique ability to self-renew, proliferate, and differentiate into effector Tcm, which suggests that this T-cell subset will be most effective and persistent upon adoptive transfer.

Thus, we first selected Tcm using the Dreg56 (anti-CD62L) biotinylated antibody and CliniMacs selection. The purified Tcm were then stimulated twice with cytomegalovirus (CMV) pp65 protein, resulting in 40% enrichment of CMV pp65 tetramer+ cells. The CMV-specific T cells were expanded more than 1000-fold following a single restimulation in rapid expansion medium, and then genetically redirected to target CD19 on acute lymphoid leukemia through lentiviral transduction with a CD19-specific chimeric antigen receptor, CD19R. Upon further stimulation and expansion of these T cells, expression of both the CD19R transgene and the endogenous CMV-specific T-cell receptor were stable as detected by flow cytometry and/or Western. Cytotoxicity assays revealed that the bi-specific T cells could efficiently and specifically kill CMV+ targets as well as the acute lymphoid leukemia cell line SupB15. Together, these results suggest that the generation of bi-specific T cells is a feasible strategy with the potential to enhance the overall antitumor potential of adoptively transferred T cells.

Isolating High-affinity T-cell Receptors for Adoptive Therapy of Tumors

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If one wants to obtain high-avidity T cells specific for tumor-associated antigens (TAA), one has to deal with the problem that such TAA often represent self-peptides derived from overexpressed proteins presented by self-MHC molecules. T cells with high-affinity T-cell receptors (TCR) specific for such ligands have been eliminated through the process of negative selection during lymphocyte development to prevent autoimmunity. Therefore, TCR affinity of remaining lymphocytes for such self-peptides is low. To efficiently generate peptide-specific T cells bearing high-affinity TCR one can tap a nonselected T-cell repertoire when the TAA peptides are presented by allogeneic MHC molecules. Several approaches have been used to obtain such allo-restricted, peptide-specific T-cell clones. We concentrated on dendritic cell (DC) priming and developed a method using RNA-loaded DC as antigen-presenting cells to tap unselected T-cell repertoires. For proof of principle, we compared self-restricted and allo-restricted priming using tyrosinase as a model TAA. Self-restricted, peptide-specific T-cell clones from healthy HLA-A2+ donors were derived using autologous tyrosinase-RNA–pulsed DC whereas allo-restricted T-cell clones from healthy HLA-A2– donors were obtained using autologous DC loaded with RNA encoding tyrosinase and allo-HLA-A2 molecules. We demonstrated that allo-restricted T cells showed functional superiority and higher functional avidities. We extended these findings by generating allo-restricted, high-avidity T cells specific for a Mart-1 peptide and a survivin peptide to show universal application.

Since adoptive transfer of TCR-transduced peripheral blood lymphocytes (PBL) provides a new therapeutic treatment our aim was to generate TCR-transgenic PBL using cloned TCR sequences. To this end, we transduced PBL of healthy donors with self-restricted and allo-restricted sequences of tyrosinase-specific T-cell clones with highest functional avidity and best capacity to kill tumors. The allo-restricted TCR-transduced PBL recognized lower peptide concentrations and therefore showed higher functional avidity compared with the self-restricted TCR-transduced PBL, demonstrating the superiority of the allo-restricted TCR.

CANCER STEM CELLS AND THE HOST RESPONSE

Characterization of the Immune Profile of Cancer Stem Cells Isolated From Human Glioblastoma

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Cancer Stem cells (CSCs) represent the most aggressive component of tumors and have been proposed as elective cellular target in the context of biological therapies such as immunotherapy. The main objectives of our project are represented by the identification of markers with immunological relevance expressed by CSCs and the validation of their role as target molecule to design immunotherapeutic protocols for glioblastoma (GBM).

We carried out a set of experiments using IF and cytofluorimetric or confocal microscopy analysis aimed at the immunological characterization of CSCs isolated from human GBM and in vitro cultured either in the presence or absence of mitogens. We found that GBM CSCs were negative or weakly positive for the expression of MHC class I or class II molecules, with only 1 out of 8 GBM CSC lines expressing high level of HLA molecules. Along this line, NKG2D ligands (MICA/B or ULBPs) were weakly or not expressed by most GBM CSCs with only 1 cell line being positive for all these molecules while significant expression of these molecules was detected on GBM tumor cell lines [grown in vitro under standard culture conditions (FBS)]. Moreover, defective expression of MHC antigen processing machinery (APM) by GBM CSC lines was observed. Up-regulation of MHC class I and of most of APM molecules was achieved after interferon (IFN)-γ treatment of CSCs, while weak or no modulation of MHC class II molecules was observed. Heterogeneous expression of MHC molecules or NKG2D ligands was also observed in tumors generated by intracranial or subcutaneous transplantation of GBM CSCs in immunodeficient mice. Notably, cancer-testis TAAs, such as NY-ESO or MAGE was weakly or not expressed by GBM CSC lines while survivin and COA-1 were detected in all these cell lines (N = 8). We carried out in vitro stimulation of peripheral blood mononuclear cells isolated from 2 GBM patients with autologous CSCs and the specific reactivity of T lymphocytes against GBM CSCs was evaluated by IFN-γ release (enzyme-linked immunosorbent spot) or cytotoxic activity (CD107a mobilization). We found that GBM CSCs, following IFN-γ treatment, can elicit an efficient CSC-specific T-cell–mediated immune response.

Taken together, these results indicate that MHC molecules and NKG2D ligands are expressed heterogeneously by both in vitro established CSC lines and in tumors transplanted in immunodeficient mice. In addition, though the expression of APM is defective in these cells, we found that GBM CSCs can be exploited to generate T-cell–mediated immune responses in at least some GBM patients.

Upregulation of Stem Cell-related Genes in Hypoxic Mammosphere Cultures

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Nonadherent mammosphere cultures have been used as a method for expansion and maintenance of mammary gland stem cells in vitro. We have observed that mammospheres cultured in hypoxic condition survived better and could be maintained at higher passage numbers compared to normoxic conditions. We sought to examine the possible involvement of stem cell regulatory genes in the maintenance of these mammospheres under hypoxic conditions. We dissociated cells from normal as well as breast cancer samples, and cultured them in suspension conditions either under normoxia or hypoxia (0.5% O_2). At 20,000 cells/ml concentration, only 6 out of 8 samples tested formed mammospheres after 7 to 10 days of culture. The number of these primary mammospheres varies from sample to sample. There was no difference in the numbers when cells from the same sample were incubated under hypoxic or normoxic conditions. Following formation of the primary mammospheres, we dissociated and subcultured them at similar cell concentrations. These mammospheres survived variable passage numbers, ranging from 2 to 10 passages. Mammospheres from sample number 9 showed the highest passage number. To investigate the level of stem cells

regulatory genes in these mammospheres, we extracted their total cellular RNA after passage 6 and screened for the presence of stem cell regulatory genes, using semiquantitative RT-PCR. Our investigation revealed that mammospheres grown in hypoxic conditions expressed higher levels of stem cell-related genes, compared to mammospheres grown in normoxic conditions. The genes found to be upregulated were jagged-1, notch-1, notch-3, musashi-1, and oct-4. This upregulation of stem cell-related genes was observed in the mammospheres formed from cells dissociated from normal as well as cancer samples. This observation suggested 2 possibilities: (1) only cells with stem cells properties have higher survival rates under hypoxic conditions; or (2) fully differentiated cells reverted to a more primitive state under conditions of hypoxia, perhaps via upregulation of stem cell-related genes, hence causing their enhanced survivability. Additional studies are required to investigate these possibilities. Nonetheless, information obtained from this study may provide useful information towards improved culture efficiencies of mammospheres and stem cell maintenance in vitro.

Regulation of Breast Cancer Stem Cells by the Microenvironment

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There is increasing evidence that breast cancers may be driven and maintained by a cellular subcomponent that exhibits stem cell properties. These properties include self-renewal, which drives tumorigenesis and differentiation, which generates the cellular heterogeneity found in the tumor bulk. These "tumor stem cells" mediate invasion and metastasis and may contribute to treatment resistance. We have developed in vitro and mouse models to investigate the influence of cells in the tumor microenvironment on cancer stem cell behavior. Humanization of NOD/SCID mouse breasts by introduction of normal mammary fibroblasts facilitates mammary gland development from normal human breast stem cells. In breast tumors, breast cancer stem cell self-renewal is stimulated by mesenchymal stem cells, which may be recruited from the bone marrow. This regulation of breast cancer stem cells by the mesenchyme is mediated by cytokines including interleukin (IL)-6, IL-8, CCL5 and CCL6. Inhibition of IL-8 signaling induces apoptosis in breast tumor cells, a process mediated by the FAS pathway. These studies demonstrate that the tumor microenvironment plays a crucial role in the regulation of breast stem cells. Interventions aimed at dysregulating microenvironmental signals may provide a novel approach to targeting cancer stem cells. Since these cells drive tumorigenesis, metastasis and mediate treatment resistance, these approaches may improve outcome for patients with advanced metastatic cancers.

CD133 as a Potential Target of Anti-cancer Stem Cell Immunotherapy: Identification of an HLA-A*02 Restricted CD133 Epitope

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Recently, we have found a small population of cells in malignant glioblastoma multiforme (GBM) that resemble cancer stem cells (CSCs). These putative GBM CSCs appear to express high levels of CD133, a surface protein that is normally absent from neuronal cells. This raises a possibility that CD133 could serve as a potential target of cytotoxic T cells (CTLs) in future GBM CSC immunotherapy. In order to find potential CTL epitopes for masses, we sought immunogenic HLA-A*0201 restricted CD133 epitopes in this study. Based on an epitope prediction, 5 potential HLA-A*02 restricted CD133 epitopes were selected for further immunologic characterizations. Among these epitopes, a ninemer demonstrated the strongest binding to HLA-A*0201 molecules. To further test the immunogenicity of this epitope, we were able to generate peptide-specific CD8+ CTLs from a normal donor by using autologous monocyte-derived dendritic cells pulsed with ILS. Moreover, monocyte-derived dendritic cells loaded with irradiated CD133-positive CSCs were to prime ILS-specific CTLs in vitro. These in vitro generated CTLs only recognized CD133 expressing HLA-A*0201+ GBM CSCs but not CD133 expressing normal neural stem cells, which lack expression of MHC class I molecules. Overall, our findings show natural processing and subsequent presentation of immunodominant CD133 epitopes in GBM CSCs, and the presence of CD8+ T cells specific for such epitope in the periphery. The results of this study have an enormous impact on current and future GBM immunotherapy since successful immunotherapy depends largely on discovery of CTL epitopes that can specifically target GBM CSCs.

CANCER AND INFLAMMATION

Recombinant Interleukin-21 Plus Sorafenib for Metastatic Renal Cell Carcinoma (MRCC): A Phase 1 Dose Escalation Study

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Introduction: Despite the benefit of tyrosine kinase inhibitor therapy for Metastatic Renal Cell Carcinoma (mRCC), durable responses are rare. Recombinant interleukin-21 (rIL-21), a cytokine that enhances CD8+ T cell and natural killer cell activity, had antitumor activity as a single agent in phase 1 studies. We conducted a phase 1/2 dose escalation study to explore rIL-21 plus sorafenib in patients with mRCC.

Methods: Subjects with mRCC (median age 63 y; ECOG 0 to 1; 0 to 1 prior systemic regimen) received sorafenib at 400 mg BID plus rIL-21 at 1 of 4 dose levels (10, 30, 40, or 50 μg/kg IV) on days 1 to 5 and 15 to 19 of a 6-week treatment course. Safety, pharmacokinetics, level of soluble CD25 (a biomarker of immune activation), and tumor response were assessed.

Results: Nineteen patients were treated. Dose-limiting toxicity of erythroderma with hand-foot syndrome was reported in 1 of 6 subjects at 10 μg/kg and 2 of 4 subjects at 50 μg/kg. The maximum tolerated dose for rIL-21 combined with sorafenib was declared to be 30 μg/kg due to increased incidence of skin toxicity at higher dose levels. Thirteen subjects completed the first 6-week treatment course and were evaluable for tumor response; all 13 received additional courses (range, 2 to 9). One subject died of progressive disease prior to finishing course 1. Five subjects were withdrawn due to dose-limiting toxicity or protocol violation and were not evaluable for response. Median progression-free survival by investigator review in the 14 evaluable subjects was 40 weeks, with a disease control rate (PR + SD) at 24 weeks of 77%. Best response included 2 PRs, 10 SD, 1 PD, and 1 subject with nonmeasurable disease by RECIST; 11 subjects had tumor shrinkage by investigator review (14% to 61%). Soluble CD25 was induced in a dose-proportional manner throughout treatment at levels comparable to rIL-21 monotherapy, suggesting that sorafenib did not negatively impact lymphocyte activation. The estimated half-life of rIL-21 did not change with dose level or re-administration; serum exposure increased in a dose-dependent manner. All subjects were negative for neutralizing anti–rIL-21 antibodies.

Conclusions: We have defined a regimen of rIL-21 plus sorafenib that can be administered in the outpatient setting and is associated with encouraging antitumor activity. No pharmacokinetic interaction between rIL-21 and sorafenib was evident, and sorafenib does not appear to diminish rIL-21–associated immune activation. Updated results from phase 1 with pharmacodynamic analysis and independent response assessment will be presented at the meeting. The phase 2 portion of the study is ongoing.

Bcl-2 Small Interfering RNA Inhibits the Growth of Human Lymphoma Transplanted Subcutaneously in Nude Mice

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Objective: Bcl-2 is the prominent member of a family of proteins responsible for dysregulation of apoptosis, and resistance to chemotherapy. In this study, we investigated whether small interfering RNA (siRNA) targeting Bcl-2 mRNA could inhibit the growth of lymphoma transplanted subcutaneously in nude mice.

Methods: We evaluated the antitumor effect of the Bcl-2 siRNA in vivo using the model of nude mice bearing Raji cells xenografts. Human Raji cells were injected subcutaneously into nude mice to establish lymphoma models. The polyethylenimine (PEI)/siRNA complex were injected into tumors. Tumor growth and tumor sizes were observed. Tissue pathological morphology was showed by hematoxylin and eosin staining. The expression of Bcl-2 mRNA and protein from the tumor mass was detected by reverse transcription-polymerase chain reaction and immunofluorescence.
Results: The tumor growth became slower in Bcl-2 siRNA-treated group, significantly different when compared to either saline controls ($P < 0.05$), or negative siRNA-treated group ($P < 0.01$). Bcl-2 siRNA significantly suppressed tumor growth on days 20, 22, and 24 ($P < 0.01$).A significant difference in median tumor weight was observed in mice treated with Bcl-2 siRNA, as compared with negative siRNA-treated mice, saline solution-treated mice ($P < 0.01$). Pathological evaluation was completed in all excised tumors. Tumor tissue of those mice treated with Bcl-2 siRNA showed apoptosis, serious necrosis and inflammatory cells infiltration. There was no change in the morphology of cells among negative siRNA-treated mice, saline solution treated mice. At Bcl-2 siRNA group, the expression levels of Bcl-2 mRNA and protein of the tumor tissue were effectively inhibited ($P < 0.01$).
Conclusions: siRNA against the Bcl-2 mRNA could inhibit the growth of human lymphoma transplanted subcutaneously in nude mice.

Bcl-2 Small Hairpin RNAs Enhance Ara-C–Induced Apoptosis in Raji Cells

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Objective: Bcl-2 is the prominent member of a family of proteins responsible for dysregulation of apoptosis, and resistance to chemotherapy and radiotherapy. In this study, we investigated whether small hairpin RNA (shRNA) targeting Bcl-2 could enhance cytarabine (Ara-C)–induced apoptosis in Raji cells.
Methods: Recombinant shRNA, targeting the coding region of Bcl-2 mRNA, expression vector with green fluorescence protein (GFP) gene has been constructed and preserved in our laboratory. Recombinant Bcl-2 shRNAs expression vector were transfected into Raji cells with Lipofectamine 2000. At 48 hours after transient transfection, the expression levels of Bcl-2 mRNA and protein were assayed by reverse transcription-polymerase chain reaction, immunofluorescence and flow cytometric method. After plasmids were transfected into Raji cells, followed by using Ara-C, the cell proliferation was determined by an MTT assay at 24, 48, and 72 hours, respectively. Apoptosis was determined by morphological observation and flow cytometric analysis.
Results: Expression levels of Bcl-2 mRNA and protein from Raji cells decreased after transfection with Bcl-2 shRNAs. There was no difference in Bcl-2 protein levels between control shRNA group and untreated cells. After transfection with Bcl-2 shRNAs, viability of cells were less than that after transfection with the cells with control shRNAs and untransfected Raji cells, respectively ($P < 0.05$). Control shRNA had no significant effect on growth of cells. Viability of cells at 48 and 72 hours after treatment with Bcl-2 shRNAs were less than that after treatment with control shRNAs and untreated Raji cells, respectively ($P < 0.05$). Control shRNA had no significant effect on growth of cells. Bcl-2 shRNA combined with Ara-C significantly inhibited the growth of cells ($P < 0.05$). There was no difference in cell survival between control shRNA/Ara-C combination and cells treated with Ara-C alone. Using Giemsa staining, cells treated with Bcl-2 shRNA combined with Ara-C at 48 hours displayed changes of apoptosis. Apoptotic rates of the Raji cells treated with Bcl-2 shRNA combined with Ara-C significantly increased ($P < 0.05$), compared with either control shRNA/Ara-C combination or Ara-C treatment cells alone.
Conclusions: shRNAs against the Bcl-2 mRNA increases Ara-C–induced apoptosis in Raji cells.

IL-29 Induces Jak-STAT Signal Transduction and can Synergize With Temozolomide and Bortezomib in Human Melanoma Cell Lines

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Purpose: Interlukin-29 (IL-29) is a member of the type III interferon (IFN) family that has been shown to have antiviral activity and inhibit cell growth. To date, expression of the receptor for IL-29 (IL-29R) has been described only in CD8+ T cells, hepatic and colonic cells and various cancers. Ligand-receptor interactions on these cells lead to activation of the Jak/STAT pathway. We tested the possibility that the IL-29R was also expressed on human melanoma cell lines and the effects it would have on the cell line.
Methods: Expression of IL-29R was evaluated using reverse transcription-polymerase chain reaction (PCR). Immunoblot analysis and flow cytometry were used to evaluate for cell signaling. The MTT and [H_3]-thymidine assays were used to measure cell line proliferation. U133 Plus 2.0 Arrays (Affymatrix) were used to screen for genes activated by treatment with IL-29. Real-time PCR was used to evaluate mRNA expression of genes up-regulated after treatment with IL-29. Finally, apoptosis was measured after treatment with IL-29 and bortezomib or temozolomide using Annexin V/Propridium Iodide staining.
Results: Both the subunits of IL-29R (IL-28R1 and IL-10R2) were expressed on the A375, 1106 Mel, HS294 T, 18105 Mel, Mel 39, SK Mel 5, and F01 cell lines. IL-10R2 was expressed on the 1174 Mel cell line in the absence of detectable IL-28R1. Incubation of A375, 1106 Mel, F01, and 1259 Mel with IL-29 (10 to 1000 ng/mL) led to phosphorylation of STAT1 (pSTAT1) and pSTAT2. pSTAT3/5 were activated in the 1106 Mel cell line, whereas the other three cell lines showed constitutive activation of pSTAT3 and no activation of pSTAT5. Treatment of IL-29 for 18 hours at 1000 ng/mL caused a two times or greater increase in more than 100 genes as compared with media treatment. The genes with the greatest increase were then tested via real-time PCR. There was an increase in expression of IFI27, RSAD2, OAS-1/2, DDX58, ISG15, IFI6, IFIT3, IFITM1, TNSF10, TFRC, S100A4, and Mx-1 and OAS mRNA in 1106 Mel, A375, and F01 over a 12-hour time period. Cell proliferation was not inhibited by IL-29 treatment in any cell line tested. IL-29 treatment induced apoptosis in the F01 cell line, but not in 1106 Mel nor A375 cell lines as measured by PARP cleavage and Annexin V staining. The combination treatment of bortezomib or temozolomide plus IL-29 resulted in increased apoptosis in the F01 cell line.
Conclusions: IL-29 receptors are expressed on the surface of several human melanoma cell lines and IL-29 treatment of these cell lines leads to signaling via the Jak/STAT pathway and increases transcription of a myriad of genes. In addition, it induces apoptosis in some cell lines. Further exploration could lead to anticancer therapies aimed at the IL-29R.

Bortezomib (B) Activity in Plasma Cell Dyscrasia (PCD) With POEMS Like Syndrome Supports an Etiologic Underlying NF-κB Inflammatory Pathway

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POEMS (polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes) syndrome is a rare multisystem disorder associated with plasma cell dyscrasia (PCD). Such symptom-complex is thought to be the direct result of overproduction of vascular endothelial growth factor (VEGF) secreted by transformed plasma cells. Although several reports revealed significant clinical benefit and response to IMIDs in POEMS syndrome lending credence to such a hypothesis, few recent reports documented no benefit and/or detriment to bevacizumab, VEGF inhibitor, raising serious doubts that VEGF is the primary etiologic pathway. We report the first case of PCD with POEMS-like syndrome with pulmonary hypertension, secondary right heart failure, and membranoproliferative glomerulonephritis that was successfully treated with single agent B, a known NF-κB pathway inhibitor. After presenting with the aforementioned signs and symptoms, a 64-year-old white man, was diagnosed with PCD (bone marrow plasma cells 15%, IgG-λ, mild-moderate anemia, normal positron emission tomography/computed tomography, normal creatinine and calcium) with ISS stage III. Due to being high risk for thromboembolic events, he was started on single agent B at 1.3 mg/m^2 days 1, 4, 8, 11 Q21 days (cycles 1 to 4) which was reduced to 1.0 mg/m^2 days 1, 4, 8, 11 Q21 days (cycles 5 to 6) due to grade 1 thrombocytopenia and neuropathy. This was followed by 3 maintenance cycles of 4 out of 5 weekly 1.0 mg/m^2. Patient's breathing was subjectively better after cycle 1 despite experiencing side effects from

his pulmonary hypertension directed treatment and subsequently improved back to his baseline by cycle 4. By cycle 2, he was found to be in nCR with complete normalization of his bone marrow and serum electrophoresis by the end of therapy. By cycle 3, his Raynaud syndrome was significantly better with less dysesthesia, which completely resolved by cycle 6. This case not only demonstrates for the first time the significant activity of B as a single agent in PCD with POEMS-like syndrome, but also serves as an in vivo experiment that supports an etiologic role for the NF-κB inflammatory pathway in POEMS syndrome.

APRIL Produced by Neutrophils Promotes Diffuse Large B-cell Lymphoma Development

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A PRoliferation Inducing Ligand (APRIL) from the tumor necrosis factor family regulates B-cell immunity. In vitro and in vivo preclinical experiments indicated that APRIL also mediates a proliferation/survival signal to B-cell lymphomas. Here, we analyzed APRIL expression in situ in human non-Hodgkin lymphomas. APRIL up-regulation was observed in 46% of diffuse large B-cell lymphoma (DLBCL, n = 312), but not in low-grade lymphomas, such as chronic lymphocytic leukemia (n = 34), marginal zone lymphoma (n = 12), mantle cell lymphoma (n = 27), and follicular cell lymphoma (n = 149). In DLBCL, neutrophils infiltrating the tumor tissue were the main cellular source of APRIL. APRIL secreted by neutrophils accumulated on tumor cells via proteoglycan binding. In addition to proteoglycans, DLBCL tumor cells expressed the canonical APRIL signaling receptor, TACI and/or BCMA, indicating that these tumor cells are fully equipped to respond to APRIL. Notably, retrospective clinical analysis with 2 different DLBCL cohorts revealed a significant correlation between high expressions of APRIL in tumor lesions and decreased disease-free and overall survival rates in patients. Hence, APRIL produced by inflammatory cells infiltrating lymphoma lesions may increase lymphoma aggressiveness and affect disease outcome (Fig. 1).

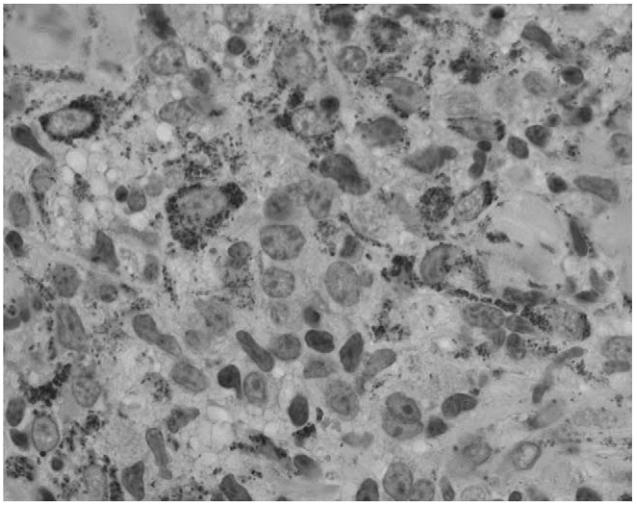

FIGURE 1. APRIL secreted by neutrophils is retained at the surface of DLBCL tumor cells, promoting lymphoma development.

Mast Cells are Essential for Polyp Formation and Colon Cancer Progression

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We have used mice conditional for the stabilization of β-catenin or defective for the adenomatous polyposis coli (APC) gene to investigate the identity and importance of tumor infiltrating hematopoietic cells in polyposis. We show that, from the onset, polyps are infiltrated with mast cells (MC) and their precursors. Both mastocytosis and polyposis were exacerbated in Rag-deficient mice that are devoid of T and B cells. Depletion of MC either pharmacologically or through the generation of chimeric mice with genetic defects in MC produced a profound remission of existing polyps.

Elevated levels of serum tumor necrosis factor (TNF)-α, interleukin (IL)-17, IL-6, and IL-1 in diseased mice, and suppression of cytokine levels in MC-depleted mice indicated a role for cytokines in polyposis. TNF-α was a major MC product. We show that MC required TNF-α for their differentiation and expansion. TNF-α was critically required for the progressive growth of adenomatous polyps. We propose that MC-synthesized TNF-α functions as an autocrine factor to amplify the local MC pool at the sites of tumor formation, while driving a cascade of events to boost the incidence and progressive growth of adenomatous polyps, the immediate precursors to colon cancer. Our more recent work shows that mast cell expansion in polyps is controlled by naturally occurring T-regulatory (Treg) cells. Our observations indicate that in a highly inflammatory microenvironment, resident Tregs lose their suppressive function. Treg functions can be recovered leading to control of cancer associated inflammation and suppression of tumor growth.

The Genetic Background of IFN-α Responsiveness: A Genome-wide Study

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The genetics of the host's response to exogenous interferon-alpha (IFN-α) may affect treatment outcome in melanoma and chronic hepatitis C virus (HCV) infection; in particular, IFN-α–treated HCV-infected African Americans (AAs) have a significantly lower chance to achieve sustained antiviral response to IFN-α than Caucasians (CAs). Concordantly, peripheral blood mononuclear cells (PBMCs) isolated from HCV-infected AAs display, upon IFN-α stimulation, impeded STAT1 phosphorylation and a dampened transcriptional pattern compared to CAs. However, the genetic and/or environmental factors responsible for this phenomenon remain unknown. Most importantly, the influence of disease status (melanoma or chronic HCV) on PBMC responsiveness shadows the interpretation of the studies done on patients' cohorts.

Here, we comprehensively performed a multistep analysis aimed at the identification of genetic markers and molecular pathways determining individual's responsiveness to IFN-α in healthy donors. Responsiveness of healthy CAs and AAs was analyzed at 3 critical levels of IFN-α signaling: IFNα-induced STAT-protein phosphorylation by flow cytometry, activation of IFN-α–stimulated genes (ISGs) by a custom-made whole-genome 36k gene expression array, and genomic-scale, 1 million SNP array (Affymetrix) for genome-wide phenotype/genotype correlations. In addition, other cytokines' signaling through IFN-α–related pathways (IFN-β, IFN-λ, IFN-γ and IL-2), and IFN-α–induced STAT2 and STAT5 phosphorylation were tested.

Surprisingly, the wide variability of the response to IFN-α observed in HCV-infected and melanoma patients was not observed when the average responsiveness of AAs was compared to CAs. Furthermore, half-maximal and maximal IFN-α–induced STAT1 phosphorylation and ISG expression patterns suggested a tight normal distribution in the response to IFN-α among CAs. On the contrary, AAs displayed a bimodal distribution in their responsiveness to IFN-α ($\times 2$ P value = 0.0003), suggesting that 2 subpopulations of AAs may be differentially affected by this cytokine. Transcriptional profiling supported the notion of a predisposition to higher responsiveness to IFN-α documentable in untreated PBMCs and enhanced by stimulation. Genome-wide profiling is under evaluation to test whether 2 genetically distinct subpopulations of AAs corresponding to the

observed phenotypes could be identified and whether such differences may similarly affect diseased populations.
Thus, HCV-infected individuals and melanoma patients may react differently to IFN-α therapy due to a dynamic balance between disease and genetic background of the host that may be only partially affected by ethnicity.

The Role of α5β1 Integrins and Mycobacterial Protein Phosphatases in Responses of Bladder Cancer Cell Lines to BCG Therapy

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Bacillus Calmette Guerin (BCG) adjuvant therapy of superficial bladder cancer is the most successful immunotherapy for solid tumors to date. However, not all patients respond favorably to therapy and BCG can secrete or express factors that will deter or retard its effects on immune response. It has previously been shown that contact between bladder cells and BCG, mediated by α5β1 integrins, is required to initiate an immune response. BCG internalization by bladder tumor cells can induce a cytotoxic response that may eradicate tumors and release tumor antigens, which could produce a specific immune response. BCG secretes protein tyrosine phosphatases (PTPs) as one of the various measures to circumvent host defenses and the hostile environment in the host cells. Our aim is to study the direct effects of BCG treatment on gene expression responses and BCG internalization of bladder cancer cell lines and to elucidate the effects of mycobacterial PTPs on tumor host cells and its responses to BCG treatment. We tested a panel of 6 bladder cancer cell lines, which differentially expressed α5β1 integrins. Cell lines with high levels α5 integrins (UMUC3, 76 ± 2.4 RFU) are able to internalize fluorescein isothiocyanate-labeled BCG more efficiently than cell lines with low levels of these integrins (SW780, 6.5 ± 1.4 RFU). Using BrDU, BCG internalization was found to correlate with cytotoxicity (mean difference: UMUC3, 8.3 ± 1.6; SW780, 0.85 ± 1.2) OligoArray hybridizations done with BCG-treated MGH cRNA showed up-regulation of various genes in inflammatory and stress responses. Of these, TNF-α and GSTT2 were further studied. When BCG interaction with MGH cells were blocked via a transwell apparatus, the expression of GSTT2 and TNF-α were significantly down-regulated with respect to control. To test for the involvement of MPTPs, both MGH cells and BCG were pretreated with 100 μM sodium orthovanadate, a phosphatase inhibitor, for 1 h before co-culture with the transwell apparatus. The previously observed down regulation was reduced, suggesting the involvement of phosphatases and was confirmed using purified MptpA. Our studies reveal that α5β1 integrins expression correlates to BCG internalization and gene expression changes following treatment. Phosphatases secreted by BCG negatively affect gene expression changes that lead to favorable BCG responses. Thus, targeting MPTPs may be an alternative approach to therapy, either by using MPTP knocked out BCG strains for immunotherapy or using purified MPTPs to reduce the activity of up-regulated kinases in a tumor microenvironment before chemotherapy. As such, studies on their potential effects on tumors and immune cells must be further characterized.

Arginase I Producing Myeloid Derived Suppressor Cells (MDSC) in Renal Cell Carcinoma are a Sub Population of Activated Granulocytes

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Arginase I producing myeloid-derived suppressor cells (MDSCs) are increased in the peripheral blood and tumors of patients with renal cell carcinoma (RCC). MDSC inhibit T-cell proliferation and cytokine production by reducing availability of L-arginine and are considered an important tumor escape mechanism. We aimed to determine the origin of arginase I-producing MDSC in RCC and to identify the mechanisms used to deplete extracellular L-arginine. The results show that human MDSC producing arginase I are a subpopulation of activated polymorphonuclear cells (PMN) expressing high levels of CD66b. Depletion of CD66b cells from peripheral blood mononuclear cells of RCC patients restores T-cell function in vitro. Activation of normal PMN induces phenotypic and functional changes similar to MDSC and promotes the release of arginase I from intracellular granules. Furthermore, high levels of free arginase I, but not arginase II, and high arginase activity were detected in plasma of RCC patients, resulting in low levels of L-arginine. Interestingly, although activation of PMN usually ends with apoptosis of these cells, there was no increase in the percentages of apoptotic cells within the MDSC as compared to autologous PMN or PMN obtained from normal controls. High levels of vascular endothelial growth factor have been associated with the accumulation of immature dendritic cells in cancer patients. However, treatment of RCC patients with antivascular endothelial growth factor antibody bevacizumab did not prevent the accumulation of MDSC. Instead interleukin-2 treatment increased the number of MDSC in blood and the plasma levels of arginase I in RCC patients. These results provide new insights on the mechanisms of tumor-induced anergy/tolerance in human cancer patients.

RLIP76: A Target for Kidney Cancer Therapy

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RLIP76 or RALBP1, a stress-responsive, multifunctional protein with multispecific transport activity toward glutathione conjugates (GS-E) and chemotherapeutic agents, is frequently overexpressed in malignant cells. Our recent studies (Singhal, et al. *Cancer Res.* 2006;66:2354; 2007;67:4382 and *J Biol Chem.* 2008;283:19714) suggest that it plays a prominent antiapoptotic role selectively in cancer cells. Despite recent improvements in drug therapy targeting kinase signaling pathways, kidney cancer still remains a deadly malignancy if not detected and removed in early stages. It is characteristically so highly drug resistant, that no effective life-prolonging regimen of chemotherapy is available for kidney cancer despite several decades of effort. Kidney cells characteristically express high levels of transporter proteins in their membranes that can contribute to drug resistance. However, the ABC transporter family proteins such as MRP1 have not been effective targets for approaches to reverse drug resistance in kidney cancer. We have previously shown that in mouse tissues including kidney, RLIP76 accounts for up to 80% of the transport of GS-E and blocking the RLIP76-mediated transport of GS-E in cells results in the accumulation of proapoptotic endogenous electrophiles and onset of apoptosis. Here, we demonstrate that when RLIP76 mediate transport of GS-E is abrogated either by anti-RLIP76 IgG or accumulation of 4-hydroxynonenal (4-HNE) and its GSH-conjugate (GS-HNE), a massive apoptosis is observed in cells. The results of our in-vivo studies demonstrate that administration of RLIP76 antibodies, siRNA or antisense to mice bearing xenografts of Caki-2 kidney cancer cells lead to near complete regression of established subcutaneous xenografts with no apparent toxic effects. These studies indicate that RLIP76 serves a key effector function for the survival of kidney cancer cells and that it is a valid target for cancer therapy. Since anti-RLIP76 IgG (which inhibit RLIP76 mediated transport) and siRNA (which deplete RLIP76) showed similar tumor regressing activities, our results indicate that the inhibition of RLIP76 transport activity at the cell surface is sufficient for observed anti-tumor activity.

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The Antiproliferative Cytostatic Effects of a Self-activating Viridin Prodrug

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Charlestown; [2]Department of Biology, Massachusetts Institute of Technology, Cambridge; MA. [3]Department of Systems Biology, Harvard Medical School; [4]Division of Signal Transduction, Beth Israel Deaconness Medical Center, Boston, MA.

Although viridins like wortmannin (Wm) have long been examined as anticancer and anti-inflammatory agents, their ability to self-activate has only recently been recognized. Here we describe the cytostatic effects of a self-activating viridin ("SAV"), which is an inactive, polymeric prodrug. SAV self-activates to generate a bioactive, fluorescent viridin NBD-Wm with a half time of 9.2 hours. With cultured A549 cells, 10 μM SAV caused growth arrest without inducing apoptosis or cell death, a cytostatic action markedly different from other chemotherapeutic agents (vincristine, camptothecin, and paclitaxel). In vivo, an SAV dosing of 1 mg/kg once in 48 h (IP) resulted in growth arrest in a murine A549 xenograft tumor model, with growth resuming when dosing ceased. Although the peak serum concentration of SAV was 2.36 μM (at 2 h post-IP injection), the concentration of bioactive NBD-Wm was only 41 nM. Therefore, the bulk of SAV was present as the inactive prodrug in serum (peak = 2.36 μM), which generated low sustained concentrations of active viridin (41 nM). SAV's cytostatic action in vivo was not entirely due to direct effects on A549 cells, since its peak concentration (2.36 μM) was modest compared to its antiproliferative IC50 (1.05 μM). In addition, this peak level was reached only once every 48 hours with a protocol that produced a cessation of tumor growth. SAV is an inactive prodrug whose slow release and cytostatic activities suggest it might be useful as a component of metronomic-based chemotherapeutic strategies.

Asbestos-induced Human Mesothelial Cell Death Causes the Release of HMGB1 and Resultant Inflammation

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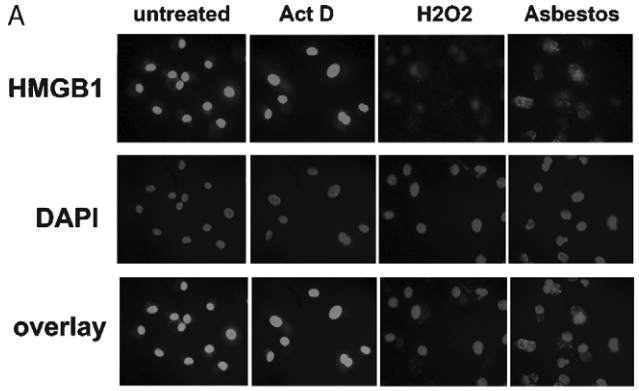
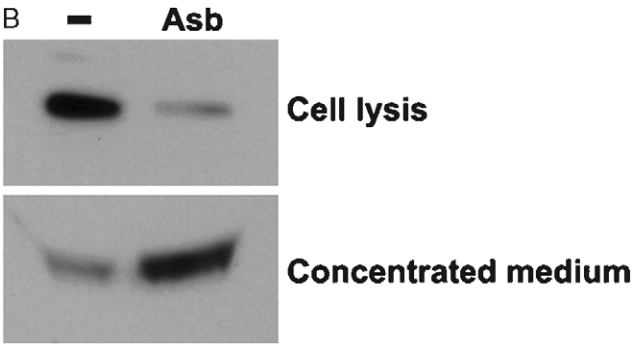
Asbestos exposure is the most common cause of malignant mesothelioma (MM), a tumor derived from the mesothelial cells that line the pleural, pericardial, and peritoneal surfaces. Asbestos carcinogenesis has been linked to the release of cytokines and mutagenic reactive oxygen species from reactive inflammatory cells. Asbestos is cytotoxic to human mesothelial cells (HM); it is unclear if this effect is also related to carcinogenesis. We found that asbestos-induced HM cell death is a regulated form of necrotic cell death, which involves poly (ADP-ribose) polymerase (PARP) activation, H_2O_2 secretion, ATP depletion, and high mobility group box 1 protein (HMGB1) release from the HM nucleus to the cytoplasm and delivery into the extracellular space. We found that the release of HMGB1 induces macrophages to secrete tumor necrosis factor-α, which on one hand protects HM from asbestos-induced cell death, and on the other hand, triggers a chronic inflammatory response that may favor tumor progression. Our findings identify the release of HMGB1 as a critical step in asbestos pathogenesis and provide a mechanistic link between asbestos-induced cell death and mesothelial cell carcinogenesis. We propose that novel chemopreventive approaches aimed at inhibiting the chronic inflammatory response induced by asbestos exposure may represent an effective means for decreasing the risk of mesothelioma and lung cancer among asbestos exposed cohorts (Fig. 1A).

HMGB1 is released from HM into the extracellular environment during asbestos exposure (Fig. 1B).



HMGB1 translocates from the nucleus into the cytosol upon asbestos exposure. The nuclei were visualized by DAPI staining. (Note that HMGB1 translocation from the nucleus into the cytosol was detected in both asbestos and H2O2 treated HM but not in actinomycin-D treated HM.)

FIGURE 1A.



HM were exposed to asbestos (5 μg/cm2) for 24 hours. Cell culture medium was collected and concentrated by ultrafiltration and tested by Western blot using a HMGB1 specific antibody.

FIGURE 1B.

CO-STIMULATION/IMMUNOREGULATION

Influence on the Mutual Expression of Costimulatory Molecules on Renal Cell Carcinoma Lines Following Stable and Transient Transfection of CD80, CD86 and B7-H1

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Within the last 2 decades, it has become evident that T-cell activation, T-cell inactivation, and T-cell death are mostly governed by the balance between positive and negative T-cell costimulatory signaling. B7-H1 (CD274) was recently discovered as a tumor-associated molecule that participates in T-cell costimulation as a negative regulator that inhibits T-cell proliferation, reduces cytokine production by T cells and promotes apoptotic death of activated tumor antigen-specific human T cells in vitro. Engagement by B7-H1 of the corresponding receptor PD-1 (CD279) on activated T cells led to reduced T-cell receptor signalling. Blocking of PD-1 ligation by monoclonal antibodies against B7-H1 reduced apoptosis of cytotoxic T lymphocytes (CTLs), augmented their expression of interleukin (IL)-2 and enhanced their killing of tumor cells. B7-H1 is aberrantly expressed on a broad range of solid tumors and recent observations have shown that tumor expression of this molecule in renal cell carcinoma (RCC) is associated with a worse prognosis for RCC patients, indicating that B7-H1 may function as a key determinant in RCCs, abrogating immune responses.

For this reason, we analyzed the expression of B7-H1 in established RCC lines and control normal kidney cells at the transcript as well as the protein levels. Unmodified RCC cells showed constitutive and rapid interferon-γ–inducible expression of B7-H1, whereas B7-H1 was not

detected in short-term cultures of normal kidney cells. Several studies could show clearly that CD80 costimulation was unable to overcome B7-H1 inhibition in mouse models, whereas B7-H1 inhibition of proliferation and function in murine CD8+ T cells was overcome by exogenous IL-2. In our former studies, we compared ex vivo expansion of CTLs from peripheral blood lymphocytes using autologous RCC cells expressing natural levels of B7-H1 with tumor cell lines genetically modified to express CD80 and/or IL-2 as stimulating cells in mixed lymphocyte tumor cell cultures. We found an important role for IL-2 in enhancing CTL proliferation and survival and the beneficial effect of IL-2 was shown to be associated with up-regulation of the antiapoptosis factor, Bcl-xL, in the CTLs (Frankenberger et al. *CCR*. 2005). In this study, we focused our analyses on transcript expression as well as surface expression of costimulatory CD80 and tumor-associated B7-H1 in our gene-modified RCC sublines. Furthermore, the mutual influence on the expression of positive and negative costimulatory molecules in these RCC lines was assessed following transient transfection of in vitro transcribed mRNA molecules coding for CD80, CD86, or B7-H1.

Clinical Activity and Safety of Ipilimumab in Combination With Placebo or Prophylactic Budesonide in Patients (Pts) With Advanced Melanoma and Brain Metastases (Mets)

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Prognosis for patients (pts) with advanced melanoma and brain metastases (mets) is poor (median survival ~4 mo). The efficacy and safety of ipilimumab, a monoclonal antibody against cytotoxic T lymphocyte antigen-4 (CTLA-4), were examined in pts with brain mets (phase II study CA184-007). Pts (treatment naive or previously treated) with unresectable stage III or IV melanoma received ipilimumab 10 mg/kg every 3 weeks (Q3W) × 4 + placebo (Group A) or prophylactic budesonide (Group B); eligible pts received maintenance ipilimumab Q12W starting at week 24. Stable brain mets at baseline were allowed, as the primary end point was safety. Brain scans were required at screening, and at subsequent time points if clinically indicated. Best overall response was assessed using modified World Health Organization criteria (complete/partial response, stable/progressive disease) by an Independent Review Committee (IRC). IRC members reviewed images (including brain scans), independently selected index lesions for tracking, and recorded all non-index lesions (including brain lesions). The first tumor assessment was at week 12. In all, 115 pts were evaluated; 12 had brain mets at baseline per the IRC: 5 in Group A and 7 in Group B. Best overall response and survival (as of December 1, 2007) were determined (Table 1). Seven pts had ongoing survival at 10+ mo. The safety evaluation included 4 additional pts with baseline brain mets per an individual IRC reviewer. Adverse events (AEs) involving the central nervous system (CNS) were reported in 5 of the 16 pts and considered drug related in 3 [one Grade (Gr) 2 headache, one Gr 1 dizziness, and one Gr 2 confusion and subsequent hospitalization]. Two pts had Gr ≥3 AEs involving the CNS (unrelated to ipilimumab): one Gr 3 brain edema (day 29) and one Gr 4 seizure (day 10). Melanoma pts with brain mets treated with ipilimumab (+ placebo or prophylactic budesonide) experienced response and survival benefit. Most treatment-related AEs involving the CNS were low grade. A study of ipilimumab in advanced melanoma pts with brain mets is ongoing.

TABLE 1.

Pt	Group	Prior Systemic Therapy?	BOR	Survival (mo)
7059	A	N	Unknown	2.7
7038	A	Y	PD	6.6
7092	A	N	PD	8.0
7056	A	Y	PD	9.3
7116	A	Y	PR	11.3+
7131	A	N	PD	11.6+
7028	A	N	SD	19.4+
7036	B	Y	PD	6.8
7130	B	N	SD	10.4+
7106	B	Y	PD	11.5+
7054	B	Y	Unknown	15.7+
7039	B	N	SD	16.4+

BOR indicates best overall response; PD, progressive disease; PR, partial response; Pt, patient; SD, stable disease.

Late-stage Tumors are Enriched in T Cells Bearing a GITR^{HI}/OX40+ Phenotype and are Effectively Treated With GITR and OX40 Agonists

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Despite evidence that tumor-specific T cells can be generated spontaneously or in response to vaccination, such T cells are often functionally inactive as a result of immunoregulatory factors produced within the tumor microenvironment. Thus a major goal of tumor immunotherapy is to define strategies capable of either directly reactivating tumor-specific T cells or inhibiting dominant suppressive mechanisms that prevent T-cell effector function(s) in vivo. GITR and OX40, costimulatory receptors belonging to the tumor-necrosis factor receptor superfamily, are known to be expressed constitutively on regulatory T cells (Treg) and up-regulated shortly after activation on effector/memory T cells. Although structurally similar, GITR and OX40 signaling appears to mediate T-cell activation via distinct mechanisms, rendering effector cells resistant to regulatory T-cell suppression or directly inhibiting Treg function, respectively. Recently we observed that agonistic antibodies and ligand-Fc fusion proteins targeting GITR and OX40 induce complete rejection of murine sarcomas when administered to late-stage tumor-bearing mice (ie, day 17 after tumor inoculation), accompanied by the development of tumor-specific CD8+ T-cell memory. Low percentages of CD4+ and CD8+ T cells that exhibit a GITR^{hi} or OX40+ phenotype were observed in the spleens of naive mice, as well as in the tumor-draining lymph nodes of sarcoma-bearing mice. However, expression of both costimulatory molecules was highly elevated on effector T cells in the progressive tumor microenvironment, peaking at approximately day 21 as determined by both flow cytometry and fluorescence microscopy. Ongoing studies to address the mechanism(s) through which therapeutic agonist reagents targeting GITR and OX40 mediate their activities will expand our knowledge of costimulatory pathways relevant to immune regulation of late-stage disease and define novel therapeutic strategies in this setting.

Autoimmunity is Associated With Clinical Benefit in Metastatic Melanoma Treated With Combination Biotherapy of IFNÁ-2B and Anti-CTLA4 Blockade

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Background: High-dose IFNα-2b (HDI) and anti-CTLA4 blockade with tremelimumab are 2 immunomodulatory therapeutic approaches that demonstrate activity in advanced melanoma. We hypothesized that the combination of these agents would effect more potent antitumor immunity and antitumor responses.

Methods: Tremelimumab was given at 15 mg/kg IV per course (12 wk). HDI was given concurrently starting with IV induction at 20 MU/m^2 IV,

5 days/week, for 4 weeks followed by maintenance with 10 MU/m^2 SC, 3 times a week, for 8 weeks per course. From course 2 onward, HDI was given only SC at 10 MU/m^2 TIW. A 2-stage design was adopted. Clinical and serologic (ELISA; DiaSorin, Stillwater) markers of autoimmunity and multiplex analysis (SearchLight Proteome Arrays, Boston, MA) of serum protein levels (IL-1a, IL-1b, IL-6, TNF-α, MIP-1b, IFN-α, EGF, IL-2R, MIP-1a, IP-10, VEGF, HGF) were measured at baseline and during therapy. Two preliminary analyses were conducted: (1) whether biomarkers were modulated by IFNα + anti-CTLA-4 and (2) whether biomarkers exhibited significant differences between biological responders (PR + SD) and nonresponders (PD).

Results: Sixteen patients (10 males, 6 females), age 32 to 75 (median 55) have been enrolled to date. All had AJCC stage IV melanoma (4 M1a, 2 M1b, 10 M1c) and all had previously received therapy for metastatic disease (range 1 to 5 prior regimens). Two patients had previously treated stable brain metastases. A total of 27 courses have been administered (median of 1 course per patient; 3 patients continue on therapy). The frequency of grade 3/4 toxicities does not exceed experience with the FDA-approved HDI regimen alone. The overall response rate is 19% (3 partial responses lasting 6+, 9+ and 9 mo). Responses were seen in both M1a (1) and M1c (2) disease. Two of the PRs were associated with clinical autoimmune manifestations (autoimmune colitis and marked vitiligo). Six patients have stable disease lasting 1.5 to 10+ months and 4 have had progression. Among 7 patients with PD, 1 had evidence of autoimmunity compared to 8 out of 9 patients with SD or PR ($P = 0.009$; Fishers' exact test). IFNα + anti-CTLA-4 therapy resulted in a significant increase of serum levels of the antiangiogenic IFN-γ inducible protein 10 (IP-10; $P2 = 0.002$) and the marker of T-lymphocyte activity IL-2 receptor (IL-2R; $P2 = 0.003$).

Conclusions: Development of vitiligo and autoantibodies is significantly associated with therapeutic benefit following IFNα-2b and anti-CTLA-4 combination biotherapy. Serum levels of IP-10 and IL-2R are significantly modulated. This study has now proceeded into a second stage in which 21 more patients will be treated.

Impairment of Secondary Antitumor Responses After IL-2/Anti-CD40 Immunotherapy: A Putative Role for Natural Killer Cells and Perforin

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We showed previously that interleukin (IL)-2 and CD40 ligation synergize for improved antitumor responses in several models of metastatic disease. Although interferon (IFN)-γ is critical for these initial antitumor responses, we showed subsequently that IFN-γ also short-circuits the efficacy of secondary antitumor responses by inducing CD4$^+$ T-cell apoptosis and impairing T-cell memory responses.

To determine the cellular mechanisms involved in the CD4$^+$ T-cell apoptosis, we analyzed secondary antitumor responses in mice deficient for perforin (Pfp), TRAIL, MRL-Fas, or IFN-γR. As expected, secondary antitumor responses were restored in IFN-γR −/− mice, confirming a role for IFN-γ in this process. Interestingly, secondary responses were also restored in Pfp mice, implicating a role for perforin in the loss of CD4$^+$ T cells and T-cell memory. In contrast, TRAIL −/− and MRL-Fas mice were indistinguishable from wild-type mice and demonstrated no restoration of secondary responses.

The depletion of CD8$^+$ T cells had no effect upon CD4$^+$ T-cell number or apoptosis after IL-2/αCD40 combination therapy. We therefore hypothesized that natural killer (NK) cells expressing perforin might be cellular mediators of the CD4$^+$ T-cell loss. IL-2 or αCD40 therapy resulted in an expansion and activation of NK cells within tumors. Furthermore, the depletion of NK cells using either asialo-GM1 or anti-NK1.1 immediately prior to and during IL-2/αCD40 therapy resulted in enhanced primary antitumor responses. These data suggest that NK cells may limit T-cell–mediated immune responses.

Although immunotherapies inducing IFN-γ expression and NK cell expansion can lead to successful primary antitumor responses, our data suggest that they may also elicit the perforin-dependent loss of CD4$^+$ T cells and impair the development of secondary memory immune responses.

Immunotherapy With Anti-CD40 and IL-2 Results in Antigen Independent CD8+ T Cell Activation In Vivo

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Our laboratory has previously shown that immunotherapy with an agonist CD40 monoclonal antibody in combination with interleukin (IL)-2 results in synergistic antitumor effects in several murine tumor models. Early studies suggested that the antitumor effects induced by this immunotherapy regimen were antigen specific in nature, and dependent upon CD8 T cells and interferon-γ. In the present study, our goal was to further characterize the effects of anti-CD40 and IL-2 immunotherapy on effector T cells, and to examine the role of antigen specificity in the efficacy of treatment. In vitro studies showed that lymphocytes isolated from treated mice were unable to proliferate in response to allogeneic mixed lymphocyte culture. Because alloreactive T cells proliferate in an antigen-specific manner, these results suggested that the antitumor effects we observed after immunotherapy might not be antigen specific in nature. With this observation in mind, we hypothesized that the antitumor effects resulting from anti-CD40 and IL-2 immunotherapy are not due to the induction of antigen specific T cells alone, but may also be due to the increased activation and killing capability of antigen-independent effector T cells. We found that immunotherapy with anti-CD40 and IL-2 results in the expansion of CD8 T cells, as well as increased production of interferon-γ. To examine the proliferative and lytic function of effector T cells generated after immunotherapy, we assessed the ability of lymphocytes from anti-CD40 and IL-2–treated mice to proliferate in response to mitogen stimulation, and to nonspecifically lyse tumor cells in vitro. We observed that lymphocytes isolated from immunotherapy treated mice displayed increased lytic function compared to nontreated controls, but did not proliferate in response to mitogen stimulation. Interestingly, we did not observe an increase in natural killer (NK) cell number or the augmented lysis of NK cell sensitive tumor targets, suggesting that the increased lytic effects we see after treatment are not mediated by NK cells. Taken together, these data suggest that treatment with anti-CD40 and IL-2 not only results in the expansion of tumor antigen specific T cells, but may also induce antitumor responses through the expansion and increased lytic function of antigen nonspecific effector T cells. Furthermore, these antigen-independent CD8 T-cell responses may contribute to the antitumor effects generated with this therapy.

Cytotoxic T Lymphocyte-associated Antigen 4 Blockade Enhances Polyfunctional NY-ESO-1 Specific T Cell Responses in Metastatic Melanoma Patients With Tumor Regression

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Blockade of the inhibitory signals mediated by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) has been shown to enhance T-cell responses and induce objective, durable clinical responses in patients with metastatic melanoma. The functional impact of anti-CTLA-4 therapy on human immune responses is still unclear. To explore this, we analyzed immune-related adverse events and immune responses in metastatic melanoma patients treated with ipilimumab, a fully human anti-CTLA-4 monoclonal antibody. We have treated a large cohort of refractory metastatic melanoma patients enrolled on 2 phase II trials of ipilimumab, sponsored by Bristol-Meyers Squibb. Patients had received a variety of prior chemotherapies and immunotherapies, including 1 patient who was previously immunized with a NY-ESO-1 protein

vaccine. Patients received an induction regimen with 4 doses of ipilimumab at 10 mg/kg given every 3 weeks or a blinded dose (0.3, 3, or 10 mg/kg) given along the same schedule. Patients were eligible for maintenance doses every 3 months if clinical benefit was observed in the absence of significant toxicity. Fifteen ipilimumab-treated patients were selected on the basis of availability of a suitable panel of specimens for immunologic monitoring, and 8 of these showed evidence of clinical benefit (partial or complete responses or stable disease for > 24 wk). Five of the 8 clinical responders had NY-ESO-1 antibody, whereas none of the 7 clinical nonresponders were seropositive for NY-ESO-1. All 5 NY-ESO-1 seropositive patients had clearly detectable CD4+ and CD8+ T cells against NY-ESO-1; one NY-ESO-1 seronegative clinical responder also had a NY-ESO-1 CD4+ and CD8+ T-cell response, possibly related to prior vaccination with NY-ESO-1. Among 5 clinical nonresponders analyzed, only 1 had an NY-ESO-1 CD4+ T-cell response and this patient did not have detectable anti-NY-ESO-1 antibody. Overall, NY-ESO-1–specific T-cell responses increased in frequency and quality during anti-CTLA-4 treatment, revealing a polyfunctional response pattern of interferon-γ, MIP-1β and tumor necrosis factor-α. We therefore suggest that CTLA-4 blockade enhanced NY-ESO-1 antigen-specific B-cell and T-cell immune responses in patients with durable objective clinical responses.

DENDRITIC CELLS

Autologous Dendritic Cell Immunotherapy Induced Increases in Serum TARC/CCL17 Levels are Associated With Progression-free Survival in Advanced Melanoma Patients

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We examined serum cytokine and chemokine changes in patients receiving dendritic cell (DC)-based immunotherapy against metastatic melanoma. Fifty-four patients received a series of subcutaneous injections of autologous DCs loaded with interferon-γ treated, irradiated pure autologous tumor cells. Serum collected at week 0 and week 4 was assayed by a qualitative cytokine/chemokine array (Raybiotech) for 42 different cytokines/chemokines. In a preliminary analysis of 20 patients evaluated by cytoarray, significant decreases ($P \leq 0.05$) between week 0 and week 4 were noted in the levels of MIP-1-δ, RANTES, angiogenin, oncostatin-M, vascular endothelial growth factor, and leptin while a significant increase was noted only for TARC/CCLl7. Although no clinical correlation could be shown for those cytokine or chemokines that decreased, the increase in serum TARC/CCL17 levels at week 4 above at levels above the median were associated with increased progression-free and overall survival for these 20 patients. Subsequently, week 0 and week 4 serum from 50 patients were then examined by enzyme-linked immunosorbent assay (ELISA) for TARC/CCL17. ELISA results demonstrated a significant association between high serum TARC/CCL17 levels at week 4 and progression-free survival ($P = 0.008$) and a trend for overall survival ($P = 0.06$). Baseline TARC/CCL17 levels were not a prognostic marker for survival however the magnitude of the change in serum TARC/CCL17 levels was a predictive marker of survival in response to the vaccine. Patients who appeared to benefit from the DC immunotherapy had higher serum levels of the chemokine TARC/CCL17 four weeks into the vaccine therapy.

Tumor Lysate Capture and Processing by Dendritic Cells: A Kinetic and an Immunofluorescence Study by Flow Cytometry and Confocal Microscopy Imaging

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A phase I to II clinical trial on melanoma patients using dendritic cells (DC) loaded with autologous tumor lysate is ongoing and a correlation has already been seen between survival and immune response. More information is, however, needed on DC antigen uptake and presentation mechanisms to improve clinical efficacy.

Methods: The kinetics of the tumor lysate capture process and its localization in DC were evaluated by cytofluorimetry, fluorescence microscopy, and fluorescence resonance energy transfer (FRET) by confocal microscopy performed using a Leica TCS SP5 apparatus, after lysate conjugation with fluorescein isothiocyanate or Alexa488.

Results: Cytofluorimetric analysis showed that lysate uptake was complete after 8-hour loading, independently of lysate concentration. During the loading period (2 to 8 h), the lysate localization presented an intensive and diffuse fluorescence within the immature DC cytoplasmic compartment. After 8 hours, lysate fluorescence was concentrated in a cloudlike perinuclear area, and small fluorescent spots were present. Specific immunofluorescence labeling and confocal microscopy imaging seem to exclude the involvement of an autophagosomy pathway. After 24-hour maturation, the contour of the fluorescent cloud was more defined, with an apparent decrease in the total emitted light. After 48-hour maturation, fluorescence intensity further decreased, with a concomitant increase in the number of the spots. In this phase, specific immunolabeling of MHCII-DR with Alexa546 was used to study colocalization with Alexa488-tumor lysate peptides by acceptor photobleaching FRET. FRET data indicate the appearance at the membrane surface of mDC of peptides loaded-MHCII-DR complex at 22 hours, with FRET efficiency (E%) that is still increasing at 48-hour maturation.

Conclusions: Our results show that tumor lysate capture by DC is a rapid process, with a maximal uptake at 8 hours from loading. The nature of the spotlike formations and cloudy area is being investigated through the co-localization of the fluorescent lysate and markers specific for lysosomes, autophagosomes, endoplasmic reticulum, and MHCII+ vesicles. Our confocal microscopy FRET data strongly suggest the presence and persistence of MHCII-peptide complexes on mature DC surface at 48-hour maturation, thus confirming DC immunogenicity.

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The Evaluation of Monocyte Derived Mature Dendritic Cells With Gene and Microrna Expression Microarrays

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Dendritic cells (DCs) are the most potent antigen presenting cells in the immune system and are a promising part of cancer vaccine therapy. The generation of DCs from peripheral blood monocytes, monocyte-derived DCs (MoDC), is the least invasive, and the most widely accepted method of producing large quantities of DCs for cancer vaccine clinical trials. Mature DCs (mDCs) are preferred over immature DCs, however, before being used clinically, the potency of mDCs must be thoroughly evaluated. Although a number of assays including ELISA, flow cytometry, and quantitative real-time PCR (qRT-PCR) can be used to measure DC potency, all are limited by the number of factors that can be analyzed. In contrast, gene and microRNA (miRNA) expression microarray analysis allows for the evaluation of all genetic information related to DC potency and function. Moreover, when combined with the traditional potency assays, microarrays may provide critical new information for improving the role of DCs in cancer vaccines. Elutriated monocytes were incubated for 3 days with GM-CSF and IL-4 and then treated for 24 hours with 3 different LPS and IFN-γ containing maturation cocktails: LPS plus IFN-γ; LPS, IFN-γ, plus TNF-α; and LPS, IFN-γ, TNF-α, plus IL-1β. The MoDCs treated with the 3 maturation cocktails were evaluated at several maturation times (0, 4, 8, 24 h), using gene and miRNA expression microarrays, qRT-PCR, flow cytometry (CD80, CD83, CD86, HLA-DR), and ELISA (IL-12p70 and IL-10). All 3 cocktails induced mDCs that expressed costimulatory molecules and CCR7 and produced IL-12p70. However, global gene expression analysis grouped mDCs according to maturation time, but not maturation cocktail. After 24 h of maturation 711 genes were up-regulated and 843 down-regulated compared with time 0 h. The greatest quantities of up-regulated genes were cytokines and their

receptors, interferon-induced proteins, metallothioneins, and proteins related with apoptosis and immune function. miRNA up-regulated after 24 hours included hsa-miR-155, 565, 578, 663, 189, 598, 452, 452*, 492, 331, and 373; down regulated miRNA included hsa-miR-517c, 569, 451, 593, 563, 55, 128a, 200b, and 122a. The genes predicted to be targeted by these mRNAs were most likely to belong to the following pathways: mitogen activated protein kinase (MAPK) signaling, G-protein signaling, cell cycle, apoptosis, inflammatory response, glucose metabolism, cell division, transcription, mRNA processing, GPI anchor binding, ubiquitin cycle, and cell redox homeostasis. In conclusion, global gene and miRNA expression analysis are powerful tools in assessing the potency of DCs and providing the information about functional aspects of DC.

IL-12 as an Immunopotency Marker for Fully Autologous Dendritic Cell Immunotherapeutics

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Argos Therapeutics has been developing the Arcelis platform based upon a proprietary dendritic cell (DC) process: postmaturation and electroporation with CD40L (PME-CD40L). During product and process development, a number of different DC maturation processes were evaluated for immunopotency based upon the induction of naive anti-MART-1 T-cell responses in vitro.[1,2] These studies showed that interleukin (IL)-12 was a critical factor for the induction of naive anti-MART CD8 T cells with the capacity for rapid expansion and polyfunctionality. In particular, IL-12 was required for (i) the induction of a CD28+ effector/memory phenotype, (ii) sensitization for interferon-γ and tumor necrosis factor-α induction, (iii) enrichment for T cells with high functional avidity, and (iv) overall clonal burst size. Having established the link between IL-12 and T-cell activity, we wished to determine whether there was a threshold amount of IL-12 required to induce the preferred T-cell response. To achieve different levels of IL-12 secretion from PME-CD40L DC, CD40L RNA was titrated into a series of DC stimulators, along with a set amount of MART-1 RNA, and the induced anti-MART-1 T-cell response was monitored. There was a linear correlation observed between the amount of CD40L RNA electroporated into the DC and the number of DC capable of secreting IL-12, and the absolute amount of IL-12 secreted per million DC. As expected, increased secretion of IL-12 resulted in improved T-cell function. However, the overall response reached a threshold whereby higher levels of IL-12 no longer improved T-cell activity. Therefore, a threshold concentration for IL-12 could be determined for optimal induced T-cell responses in this in vitro model system, representing an immunopotency marker. Argos Therapeutics is currently in phase II studies in renal cancer and HIV using fully autologous Arcelis DC. To confirm the potential value of IL-12 as a potency marker, spontaneous IL-12 secretion will be determined from postthaw DC products administered in the clinic, and compared to immunomonitoring data and clinical activity.

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Influence of Platelets on the Activation and Function of Sipuleucel-T, an Investigational Cellular Immunotherapy for the Treatment of Prostate Cancer

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A hallmark of many antigen presenting cell (APC) based cellular immunotherapies that are being studied to treat cancer, including dendritic cell therapies, is the need for antigen loading and in vitro activation of APCs during the culture period. It is often difficult to identify the specific factors that contribute to optimal antigen loading and activation of autologous APCs during cultivation. We have sought to identify these factors during cell process development, to help ensure reproducible manufacturing of product from any donor.

Sipuleucel-T is an investigational immunotherapy product for the treatment of prostate cancer that is designed to generate an immune response to prostatic acid phosphatase (PAP). Briefly, sipuleucel-T is manufactured from peripheral blood mononuclear cells (MNCs) collected by leukopheresis and enriched through buoyant density separations. MNCs are loaded with antigen and activated during a 2-day culture with PA2024, a fusion protein consisting of PAP and granulocyte macrophage-colony stimulating factor. Characterization studies of healthy donor MNCs in the sipuleucel-T culture showed that APC performance was influenced by platelet (PLT) content. While all products derived from healthy donor MNCs had increased CD54 expression, which has been shown to be an activation marker for sipuleucel-T, the level of APC activation was reduced with increased PLT content. Therefore, studies were designed to further evaluate the effects of PLTs on the in vitro antigen loading and activation of APCs. Following culture, manipulated MNC pools with decreased or increased PLTs were evaluated against their autologous controls for the expression of APC activation molecules, allostimulatory capacity, PAP-specific antigen presentation, and the production of APC activation-associated chemokines.

PLT culture concentration directly effected the activation and function of APCs. APC activation, as measured by CD54 expression, is reduced with increased PLT content in culture. Similarly, allostimulatory capacity was reduced with increased PLTs; however, the assay proved to be less sensitive than other functional readouts. PAP-specific antigen presentation activity, measured using a PAP-specific HLA-DRβ1-restricted T cell hybridoma, was decreased with increased PLTs. Additionally, 2 APC activation-associated chemokines commonly produced during culture, IP-10 and MIP-1β, were also reduced with increased PLTs. Residual antigen in the culture supernatants indicate that elevated PLTs may be associated with decreased antigen uptake. These data suggest that PLT levels should be measured when optimizing APC culture conditions, and that multiple functional and activation readouts should be used to assess APC performance accurately.

Phenotypic and Functional Analyses of Standard And Fast Dendritic Cells (DC) for DC-based Cancer Immunotherapy

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Dendritic cell (DC)-based vaccines have been shown to effectively induce high numbers of circulating tumor-specific T cells, but their production for clinical use is time consuming and laborious. Standard DCs are frequently derived from peripheral blood monocytes after culture them in granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 for 5 days in presence of a maturation cocktail of proinflammatory cytokines: IL-1α, IL-6, tumor necrosis factor-α, and prostaglandin E_2, for additional 48 hours. Recently, it has been published that monocytes cultured in GM-CSF and IL-4 for 24 hours and maturated with the cocktail mentioned above for additional 24 hours, lead to obtain fast DCs (herein named Fast-DCs). Although Fast-DCs may be suitable to be used as antigen vehicle for cancer immunotherapy, their use for this purpose is still to be demonstrated. In this work, a comparison of the surface expression of DC differentiation markers and co-stimulatory molecules (CD83, CD80, CD86, CD209, CD38, HLA-DR); the expression of the receptor for the CCL19/CCL21 chemokines (CCR7); and functional analyses such as priming of naive T-cells, cytokine production by CD4 and CD8 T cells and generation of cytotoxic CD8 T cells were assessed side by side using standard DC and Fast-DCs as APCs. Despite been described that the incubation of standard DC with recombinant human interferon-γ induces expression of CD38 and CD83 this was not observed in Fast-DCs, however, the expression of all surface markers analyzed was very similar between both

cellular types and furthermore, upon maturation the induction of CCR7 was higher in Fast-DCs than in standard DCs. On the other hand, the cellular yield was remarkably reduced in standard DC. Fast-DCs were suitable for expansion of CD8 cytotoxic T-cell precursors specific for an HLA-A2 restricted peptide from melanoma from naive individuals and efficiently primed CD4 T cells specific for defined antigens from naive individuals. Altogether our results suggest that Fast-DCs may be suitable for priming T cells and for efficient antigen delivering of DC-based cancer vaccines.

Blockade of Transforming Growth Factor Beta Receptor I Enhances the Efficacy of Dendritic Cell-based Immunotherapy of Established Breast Tumors

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One of the many tumor-promoting effects of transforming growth factor (TGF)-β is its ability to inhibit antitumor immunity, particularly by impairing dendritic cell (DC) function. Thus, tumor-derived TGF-β represents a major obstacle in the generation of effective antitumor immune responses by DC-based immunotherapy. Inhibition of the TGF-β receptor type I (TβRI/ALK5) has recently emerged as a safe and effective strategy to overcome TGF-β–mediated tumor promotion. Therefore, we hypothesized that systemic blockade of TGF-β signaling using an orally bioavailable ALK5 kinase inhibitor would augment the efficacy of DC-based immunotherapy of breast cancer. Mice bearing established 4T1 mammary tumors and receiving the small molecule ALK5 kinase inhibitor, SM16 in their diet were vaccinated with tumor lysate-pulsed, tumor necrosis factor-α matured, bone marrow-derived DCs. The data indicate that SM16 can safely and effectively suppress primary tumor growth and metastasis formation. Combination of SM16 therapy with DC vaccination resulted in primary tumor regression in several mice that correlated with increased T-cell infiltration of the primary tumor and enhanced in vitro interferon-γ production and tumor-specific cytolytic activity by splenocytes. Furthermore, a suboptimal dose of SM16 that failed to control primary tumor growth on its own also augmented the efficacy of DC vaccination. Taken together, these findings suggest that blockade of TGF-β signaling using an ALK5 kinase antagonist may be an effective strategy to bolster the efficacy of DC-based cancer vaccines.

ENDPOINTS, RESPONSE CRITERIA FOR CLINICAL TRIAL DESIGN

Identification of Antibody Responses Induced in Patients With Castration-resistant Prostate Cancer (CRPC) Receiving GVAX Immunotherapy for Prostate Cancer

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Introduction: GVAX immunotherapy for prostate cancer is composed of 2 allogeneic prostate carcinoma cell lines (PC-3 and LNCaP) that have been modified to secrete granulocyte macrophage-colony stimulating factor. Completed phase 2 trials include a multicenter phase 2 trial, G-0010, in patients (pts) with castration-resistant prostate cancer (CRPC). The subset of pts in G-0010 who received doses comparable to the dose used in ongoing phase 3 trials (n = 22) showed median survival of 35.0 mo.

Methods: Immunotherapy-induced antibody (Ab) responses were evaluated in 14 pts from G-0010 whose actual survival exceeded that predicted by the Halabi nomogram using 3 methods: (i) serological analysis of gene expression (SEREX), (ii) protein chip analysis, (iii) screening predefined prostate cancer antigens (Ags). Ab responses observed in at least 2 of these 14 pts were then further examined in all evaluable G-0010 pts (n = 65). Ab responses were evaluated for potential association with survival using the Cox regression model, adjusted for prognostic factors and dose group.

Results: Analysis of Ab responses in 14 CRPC pts yielded 411 candidate Ags of which 93 were seen in ≥ 2 pts. Preliminary data from all evaluable G-0010 pts suggests that Abs to protein FLJ14668, neuronatin, cardiolipin, and the PC-3–derived HLA-A24 may be associated with survival independently of treatment duration and prognostic factors. For example, pts with Ab to protein FLJ14668 (n = 34) had a median survival of 43 mo versus 21 mo in Ab-negative pts (n = 31), HR = 0.34, $P = 0.002$. Among HLA-A24 haplotype-negative pts, the HLA-A24 Ab-positive pts (n = 30) had a median survival of 43 mo versus 18 mo in Ab-negative pts (n = 28), HR = 0.53, $P = 0.05$.

Conclusions: GVAX immunotherapy for prostate cancer induces a polyvalent IgG Ab response to a broad panel of immunotherapy-derived antigens. The majority of proteins targeted are pt-specific; however, a smaller group of higher frequency Ab targets were identified. Abs to HLA-A24, neuronatin, cardiolipin, and FLJ14668-specific IgG may be associated with observed survival. Phase II immunomonitoring studies are designed to identify Ab candidates that will be evaluated prospectively in 2 ongoing 600-pt phase 3 trials of GVAX immunotherapy for prostate cancer with the goal of identifying potential biomarkers of response.

MGMT Promoter Methylation in Sporadic Colorectal Cancer

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Background: DNA methylation alteration has been proposed as a candidate molecular mediator of field defect that cause sporadic colorectal cancers. The DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) is often methylated in colorectal cancer. We hypothesized that MGMT methylation could be one of the mediators of field cancerization in the colon mucosa.

Methods: We studied MGMT promoter methylation in tumoral and nontumoral adjacent mucosa from 86 colorectal cancer patients by methylation-specific polymerase chain reaction. Statistical tests were 2-sided.

Results: MGMT promoter methylation was present in 89.3% of the tumors. Patients whose cancer had MGMT promoter methylation also had fewer MGMT promoter methylation in apparently normal adjacent mucosa 28.5%.

Conclusions: Some colorectal cancers start from a field defect defined by epigenetic inactivation of MGMT. Detection of MGMT promoter methylation status may ultimately be useful in risk assessment for colorectal cancer.

Overall Survival (OS) and New Patterns of Response in Patients (Pts) With Advanced Melanoma Treated With Ipilimumab

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Ipilimumab, a cytotoxic T lymphocyte antigen-4 (CTLA-4) monoclonal antibody, administered at 10 mg/kg to 155 melanoma patients (pts) (phase II trial CA184-008) resulted in modified World Health Organization (mWHO)-classified tumor responses and a median overall survival (OS) of 10.2 months (95% confidence interval 7.3, not reached). mWHO criteria may not capture its full clinical benefit. OS by response per mWHO or novel efficacy end points was examined. Previously treated advanced melanoma pts received 10 mg/kg of ipilimumab every 3 weeks (Q3W) × 4; eligible pts received 10 mg/kg maintenance ipilimumab Q12W starting at week 24. The primary objective was best overall response rate. OS was a secondary end point. Response was assessed using mWHO [complete/partial response (CR/PR), stable/progressive disease (SD/PD)] by an Independent Review Committee. Efficacy was measured in some pts after mWHO PD if they did not receive other therapies. Novel response end points (Hodi FS, et al. *ASCO*. 2008:abst3008) tracked total tumor burden over time when tumor shrinkage occurred after mWHO PD and captured 4 response patterns: (1) response in baseline lesions; (2) ''stable disease'' with slow, steady decline in total tumor burden; (3) response after initial increase in total tumor burden; (4) response in index + new lesions after the appearance of new lesions. There were no mWHO CRs. Median OS follow-up was 9.5 months. Median OS for pts with mWHO PR/SD (n = 42) has not been reached with only 5 deaths (11.9%). Pts with mWHO PD who then experienced tumor shrinkage per the new efficacy endpoints (n = 16) have not reached a median OS; 2 (12.5%) died. Median OS for pts with PD by both end points (n = 60) was 6.8 months (95% confidence interval, 5.5-9.3); 42 pts (70%) died. OS follow-up is ongoing. Similar OS benefit was observed in pts with mWHO PR/SD and pts with tumor shrinkage per the novel response end points (despite being assessed as mWHO PD). These data suggest PD by mWHO in ipilimumab-treated pts may not indicate drug failure. The 4 response patterns likely contribute to OS.

A Phase I Trial of Tumor Associated Antigen-pulsed Dendritic Cell Immunotherapy for Patients With Brain Stem Glioma and Glioblastoma

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Our past phase I and II immunotherapy trials (Yu J, et al. *Cancer Res.* 2001;61:842–847 and 2004;64:4973–4979) have focused on patients with malignant glioma and have demonstrated efficacy in generating a tumor-specific immune response. The goal of this study is to use tumor-associated antigens (TAA) known to be expressed on gliomas and pulse dendritic cells with these antigens in an MHC compatible fashion. In this phase I trial, sponsored by Immunocellular Therapeutics, HLA-A1 and/or HLA-A2–positive patients with newly diagnosed or recurrent brainstem glioma or glioblastoma were eligible. Specific TAA were loaded on dendritic cells prior to frozen storage. Leukapheresis was performed on patients between days −28 to −14 and used to isolate mononuclear cells. The isolated mononuclear cells were differentiated into dendritic cells in culture, pulsed with tumor peptide, and then administered on days 14, 21, and 28. The anticipated mononuclear cell yield from leukapheresis was 5×10^9 cells. Fourteen patients were enrolled between November 2006 and July 2008 with 1 screen failure due to positive antinuclear antibodies. The median patient age was 47 years (range, 26 to 65) and patients had a median Karnofsky performance status of 90% (range, 90 to 100). All patients had supratentorial glioblastoma multiforme and underwent complete surgical resection before initiation of temozolomide and radiotherapy (2 patients also received bevacizumab and 1 patient had a gliadel wafer). They were successfully treated by intradermal vaccine injections every 2 weeks × 3 and followed by adjuvant chemotherapy. Our preliminary data on 10 patients and 28 courses of dendritic cell vaccines demonstrate zero grade 3/4 toxicities that were attributable to the vaccination. Seven patients continue to have stable disease (ranging from 23 to 81 wk), 3 patients have tumor progression. This phase I study demonstrated the feasibility, safety, and bioactivity of a TAA-pulsed dendritic cell vaccine for patients with glioblastoma. For the first time, we demonstrated the ability of an active immunotherapy strategy to generate specific TAA-targeted cytotoxicity in brain tumor patients. Based on our preliminary data, we believe that pulsing autologous, peripheral blood dendritic cells with tumor peptides and reinjecting them into the patient, should help to prime the cellular immune response to the intracranial tumor and potentially generate a long-lived cytotoxic response.

Improving T Cell Assays Using HLA Class I or Class II Mono-specific APCs/Targets

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Introduction: The accurate and specific analysis of endogenous and vaccine enhanced T-cell responses is an important area of immunological and clinical research.

However the standard assays can be relatively insensitive, of limited reproducibility and cumbersome to perform. There is also a need for a large number of different antigen-specific cells (APCs)/target cells to cover the range of HLA types in differing patient populations.

With the aim of improving the accuracy of these assays we have developed a novel approach using HLA monospecific APCs/target cells. These can be used in a variety of immunological assays including enzyme-linked immunosorbent spots (ELISPOTs), intracellular cytokine assays, functional lytic analyses, and controlled T-cell expansion.

Method: Using CD20+ HLA− T2 cells, we have attached biotinylated HLA class I and II/peptide complexes using an antibody-streptavidin fusion protein binding to CD20. This immobilizes a controlled number of a chosen HLA peptide complex on the cell surface, producing an APC/target cell that is monospecific for that combination. This allows the production of large numbers of APCs/target cells that are reproducible in their characteristics and E:T ratios.

We have used these cells as APCs/targets in a number of immunological assays. In ELISPOTs and intracellular cytokine assays, they can replace the endogenous peptide-pulsed B cells, while in T-cell functional assays they can replace a panel of HLA class I-specific target cells lines such as T2 and CIR derivatives and of course can be used for any HLA allele selected.

Results: The attachment of the HLA class I/peptide complex gives high levels of activity in ELISPOT, intracellular cytokine staining and functional killing assays with T cells only of that specificity.

The results of the T2 cells coated with recombinant HLA-A2/peptide complexes compare accurately with those obtained by conventional peptide pulsing.

Data will be presented showing that T2 cells can also be used as APCs/target cells for other HLA class I alleles and for HLA class II.

Conclusions: This simple system allows the production of identical APCs/target cells that are reproducible in their immunological characteristics.

The advantages of this system include:

1. HLA monospecific APCs/targets can be made, simply, rapidly and reproducibly to any chosen HLA class I or class II/peptide combination.
2. Only a single cell line needs to be kept in culture for use irrespective of which HLA types the investigators wish to examine. The APCs/target cells will have the same baseline characteristics, whatever the choice of HLA/peptide complex.
3. The absence of other HLA class I or class II/peptide complexes on the APCs/target cells allows for a reduction in nonspecific signal.

Overall, these cells should improve the quality of T-cell assays and be simple, quick, and reliable to use.

Monitoring Immune Responses in Clinical Trials and Animal Studies

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Monitoring immune responses in the course of clinical vaccine trials and animal models of disease is widely used to assess the efficacy of immunotherapy. The enzyme-linked immunosorbent spot (ELISPOT) assay has gained increasing popularity, especially as a surrogate measure for cytotoxic T lymphocyte (CTL) responses. Previously, a human interferon (IFN)-γ ELISPOT assay was validated by our laboratory to assess CTL reactivity to specific peptides. This assay has been primarily used for the detection of T-cell responses against vaccine components by using peptide pulsed antigen-presenting cells as surrogate T-cell targets. Because immunotherapies and different cancer vaccine formulations can include whole proteins, antigen-pulsed dendritic cells or whole tumor cells in combination with various adjuvants and carriers, the laboratory has developed and validated additional modified ELISPOT assays to monitor immune responsiveness in samples against antigens other than 9 to 10 mer HLA.A2 binding peptides. These include HLA.A2 peptides > 10 mer, non-HLA.A2 binding peptides, whole proteins, patient tumor cells, whole or lysed tumor cells, or other stimuli when tumor specific antigens have not been fully identified. Different modifications of the IFN-γ ELISPOT assay developed by LCMI include: 1) whole protein ELISPOT assay to monitor immune responses to whole protein vaccinations, and sensitivity of this assay was further enhanced by optimizing the use of autologous DC as antigen presenting cells; 2) autologous tumor ELISPOT assay to test the direct reactivity of patient T cells against autologous tumor cells. These modifications can also be applied to monitor different cytokines or immune proteins beyond IFN-γ. LCMI has also modified 2 different assays to measure cell-mediated cellular cytotoxicity. These assays are a granzyme B ELISPOT, as a surrogate measure of natural killer and CTL cytotoxicity, and a flow cytometric-based cytotoxicity assay that simultaneously assesses target cell death by measuring target binding of the apoptosis marker Annexin V, and cytolytic effector cell activity and frequency by effector expression of the degranulation marker CD107a. Additionally, we have extended the ELISPOT assays to assess immune responses in different murine models. ELISPOTs for murine interleukin (IL)-2, IL-5, IL-17, IFN-γ, granzyme B, granulocyte macrophage-colony stimulating factor, and MCP-1 have been optimized. In this presentation, we report application of these assays for monitoring cancer vaccine trials and murine studies.

ENHANCING CANCER VACCINES

Selection of Ideal Particle Size for W/O Emulsions Based Vaccine

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Developing stable formulations is generally a common target to obtain efficacious emulsified vaccines. Process used to perform water in oil (W/O) emulsions is not all equivalent regarding vaccines' physicochemical parameters. Equipment generally used to realize emulsified vaccines can be high shear mixers, vortex, syringes, or T connectors. Applied process has a direct impact on quality, stability, and particles size of dispersed phase.

In this work, we have developed and characterized different production strategies, adapted to clinical development stages. The adjuvants used were Montanide ISA 51 and Montanide ISA 720. Viscosity, stability, and droplets size measures were analyzed to select the process enabling to obtain standardized and reproducible W/O emulsions.

Mice vaccination was carried out to determine the influence on immune response of diverse emulsions quality made by these different equipments. A broad particle size range, from 1 μm to 10 μm, was selected as a build model. Specific parameters such as antibody subclasses production and induced seric interleukins were followed and discussed.

Mechanical Disruption of the Primary Tumor Using Biocompatible Magnetic Beads in Combination With Immunotherapy Allows for Systemic Anti-tumor Responses in Metastatic Breast Cancer

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One of the challenges in the treatment of cancer is the eradication of systemic metastatic disease. Problems associated with current cancer therapies (ie, surgery and cytoreductive agents) include the removal of the antigen pool and potential destruction of immune effector cells. Other procedures under study (ie, cryoablation), aimed at targeted ablation of the primary tumor, show limited immunological effects. Therefore, to increase antitumor efficacy we developed a means to cause mechanical disruption to the primary tumor, followed by immunotherapy. Ferrous particles were injected as biocompatible magnetorheological fluids (MRF) into an accessible, orthotopic primary breast carcinoma, and then subjected to a magnetic field to change the MRF from a liquid to a semisolid state. We postulated that this treatment will result in marked tumor death by mechanical injury, disruption of the tumor's architecture and induction of "danger" signals: production of proinflammatory cytokines and chemokines with recruitment of dendritic cells. We have previously demonstrated that the combination of an agonist antibody to CD40 and interleukin-2 resulted in synergistic responses in advanced tumor models by activating dendritic cells and T cells. Therefore, we used MRF in combination with these immunomodulating agents at suboptimal dosages to further promote systemic immunity to tumor antigens. Mice that received MRF and magnet treatment did not show any sign of discomfort or toxicity. Histological examination of tumors treated with daily application of a magnetic field showed increased tumor death, and slower tumor growth in comparison to control groups. Tumor destruction was accompanied with increased production of pro-inflammatory cytokines (interleukin-6) and chemokines (CCL4 and CCL5) in the tumor site. We also observed increased recruitment of activated dendritic cells (ie, MHC II+, CD83+) to the tumor site. We then applied this treatment with or without immunotherapy to determine if systemic antitumor effects could be attained. Tumors were inoculated on both sides of the mammary fat pad and only 1 tumor (direct) was treated with MRF and magnetic field followed by systemic immunotherapy. We observed suppression of tumor growth on the contralateral side only in groups receiving low doses of immunotherapy in combination with the MRF treatment. These data suggest that MRF with magnetic field application and immunotherapy can induce a systemic antitumor immune response and therefore may be used to target metastatic disease.

Impact of Tumor Antigen Loading Of Dendritic Cells on CD8+ and CD4+ T Cell and NK Cell Responses

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Hepatocellular carcinoma is one of the most common and deadly cancers in the world and affects over 600,000 individuals annually. We are investigating immunotherapy approaches for hepatocellular carcinoma, including dendritic cell (DC)-based vaccines. DC vaccines have shown limited clinical efficacy in first generation trials, hence there is an urgent need for newer approaches that can improve outcomes. Here, we examined DC, which have been loaded with multiple, defined tumor-associated antigens by different antigen uptake pathways. The "self" tumor antigens α-fetoprotein, Glypican-3, and MAGE-A3 were tested. We determined the subcellular sites of tumor antigen localization and tested resultant T-cell responses. We used the following clinically relevant antigen loading strategies: peptide pulsing, protein pulsing, and adenovirus (AdV) transduction of DC, as well as their combinations. Antigen delivered via AdV transduction results in antigen synthesis in the cytoplasm. Exogenous protein loading of DC results in antigen, which appears to be in endosomes. These modes of antigen uptake differentially promote activation of antigen-specific CD4+ and CD8+ T cells, as well as have different impact on natural killer cells and regulatory T cells. DC can multiply simultaneously loaded, resulting in several specificities of antigen-specific T cells. Differences in antigen loading have broad impact on T-cell expansion, differentiation, epitope hierarchy, and skewing of CD8+ versus CD4+ T cells. Understanding

the impact of tumor antigen processing will allow for optimal DC vaccine preparation and rational design of resultant immune responses for immunotherapy of cancer.

The Immune System Strikes Back: Cellular Immune Responses Against Indoleamine 2,3-dioxygenase

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The enzyme indoleamine 2,3-dioxygenase (IDO) has a vital immunosuppressive function in the immune system, including suppression of anticancer immune responses. IDO is expressed in the tumor microenvironment as well as in antigen-presenting cells in tumor-draining lymph nodes, where it can create a tolerogenic microenvironment. Moreover, cancer cells may express high levels of IDO, strongly emphasizing the relevance for IDO expression in negatively regulating anti-cancer immune responses. In the present study, we set out to scrutinize whether IDO may be a subject to immune responses. Our data demonstrate that spontaneous specific cytotoxic T-cell responses against IDO-derived HLA-restricted peptides are elicited in cancer patients. We unequivocally demonstrate that these IDO-reactive T cells are indeed peptide specific, cytotoxic effector T cells. Most remarkable, IDO-reactive T cells are able not only to recognize and kill tumor cells, but also IDO-expressing dendritic cells, that is, the major immune suppressive cell population during T-cell priming. Consequently, as emerging evidence suggests that IDO constitutes a significant counter-regulatory mechanism induced by clinically relevant proinflammatory signals, IDO-based immunotherapy holds the promise to boost anticancer immunotherapy.

Cellular Immunotherapy and Immune Regulation in Ovarian Cancer

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While there is an increasing consensus that active immunotherapy or antitumor vaccination should be supported by selective and efficient prior depletion of tumor-associated regulatory T cells (Treg), there is also a new appreciation that dendritic cell (DC) vaccination itself may induce or expand Treg, promoting tumor-specific tolerance. Supporting the clinical observation that vaccination with cytokine-matured DC expands Treg in myeloma patients, we have found that ovarian tumor antigen-loaded DC matured with a standard cytokine cocktail [tumor necrosis factor, interleukin (IL)-1 and prostaglandin E_2] activate and expand CD4+foxp3+ Treg in vitro. It is thus probable that DC activation of antitumor effector T-cell responses would be seriously compromised. For DC vaccination to be clinically effective, the new challenge is to identify alternative pathways of DC and T-cell differentiation that bias T-cell responses away from Treg homeostasis and in favor of active antitumor immunity.

We have found that IL-15 treatment of DC and/or responder CD4+ T cells specific for serine protease ovarian tumor antigens diminishes T-cell foxp3 expression and Treg activity, with resultant reciprocal enhancement of helper T-cell function and tumor antigen-specific CD8+ cytotoxic T-cell responses. We have also shown that IL-1 can antagonize IL-2–driven human Treg expansion, subverting responses to the reciprocally regulated Th17 phenotype. IL-1 conditioning also diminished CD4+ T-cell CCR4 expression. As CCR4 is the receptor for CCL22, which is responsible for Treg homing in ovarian cancer, these results suggest that IL-1 would not only reduce Treg function, but also inhibit trafficking of foxp3+ Treg in the tumor microenvironment. Both IL-15 and IL-1 may thus regulate key points of cellular immune differentiation that are critical for the success of DC vaccination or adoptive T-cell immunotherapy. Collectively, these results support the developing consensus that Treg and Th17 differentiation and expansion are reciprocally regulated, and suggest that subversion of Treg responses in favor of Th17 responses may have therapeutic benefit for cellular immunotherapy of ovarian cancer. Apart from inhibition of Treg activation, other benefits may accrue. First, IL-15 conditioning yields a dramatic increase in T-cell expression of IL-17F, which has anti-angiogenic activity, and may thus have therapeutic value. Second, IL-15–driven CD4+ Th17 responses correlate with enhancement of CD8+ T-cell cytotoxicity. Third, Th17 responses are less sensitive than Th1 responses to Treg suppression, which may remain a barrier to immunotherapy in ovarian cancer patients, even in those pretreated with cyclophosphamide or ONTAK to deplete tumor-associated Treg.

Shikonin Enhances the Efficiency of Gene-based Vaccine Via Induction of Rantes and Recruitment of Dendritic Cells

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Shikonin, a chemically defined, potent immunosuppressive phytocompound isolated from Lithospermum erythrorhizon, has been shown to confer a variety of pharmacological activities, for example, anti-inflammatory, wound-healing, and antitumor activities. Dendritic cells (DCs) are dedicated antigen-presenting cells that play a major role not only in the initiation of T-cell–mediated immunity but also in cross-talks between the innate and adaptive immunities. DCs act as sentinel cells, for uptake and processing of foreign antigens, they then traffic to the draining lymph nodes through nonlymphoid peripheral tissues. There is accumulating evidence that the cell density and the cell differentiation and maturation status of skin DCs may greatly contribute to the efficiency of various gene-based strategies currently under development. In this study, we investigated how the anti-inflammatory activities of shikonin are involved in the augmentation of various cellular and molecular functions of DCs, including the recruitment, maturation, and migration of DCs, therefore, shikonin could overcome some of the limitations of DNA vaccine and lead the improvement of the efficiency of DNA vaccine.

Our study show that shikonin can suppress phenotypic and functional immature DCs maturation, subsequently alter their chemotactic response to CCL21, attenuate the migratory activity of matured DCs homing to lymphoid tissue and promote leukocytes recruitment by Rantes induction from keratinocytes.

To our knowledge, mechanistic and detailed molecular studies of shikonin or other phytocompounds on the enhancement of leukocytes recruitment to a specific targeted tissue have not been previously reported. Putting all findings together, we hypothesized that shikonin can provide a microenvironment for sequestering immature DCs into a concentrated high cell dose population in specific tissue and synchronize DCs into immature status, which is required in terms to create clinical opportunities for possible application of potential adjutants for DNA vaccine.

Evaluation of Interleukin-13 Receptor (IL-13R) Overexpression in Human Thyroid Cancers

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It is predicted that nearly 37,340 new cases of thyroid carcinoma will be diagnosed in the United States in the year 2008. This cancer strikes the younger population at a high rate, 2 out of 3 cases are identified in people between the ages of 20 and 55. Thyroid carcinoma is mostly cured by surgery and radiation therapy, but there are limited options for treating advanced stage and anaplastic carcinoma. Previously, we have demonstrated over expression of the interleukin (IL)-13R in a variety of human cancers including brain, AIDS-associated Kaposi sarcoma, squamous cell carcinoma of head and neck, and ovarian carcinoma. We have also demonstrated that overexpression of IL-13R sensitizes

tumors to receptor-directed therapeutic agents such as a chimeric fusion protein consisting of IL-13 and a truncated pseudomonas exotoxin (IL-13PE). This reagent has demonstrated a significant antitumor activity in various animal models of human cancer. In the present study, we have determined the expression and structure of IL-13R complex in human thyroid carcinoma specimens by triple immunohistochemical immunostaining for IL-13Rα1, IL-13Rα2, and IL-4Rα chains using highly sensitive and photostable fluorescence-labeled antibodies. Our initial results in 153 thyroid cancer specimens and 42 normal thyroid specimens indicate that overall 60% of tumor specimens strongly overexpress IL-13Rα2 chain. When analyzed by pathological subtypes, interestingly, 70% of the 67 papillary thyroid cancer specimens overexpressed IL-13Rα2 in comparison to 54% (6 of 11 cases) anaplastic thyroid carcinoma, 44% (4 of 9 cases) medullary cancer, and 40% (6 of 15 cases) follicular cancer specimens. Only 10% (4 of 42 cases) normal thyroid specimens showed modest staining for IL-13Rα2. In situ hybridization analysis for IL-13R RNA in tumor and normal specimens corroborated immunohistochemical results, confirming the expression of type I IL-13R in thyroid cancer at protein and mRNA levels. These studies suggest that IL-13Rα2 expression in thyroid cancer may be useful for receptor-targeted therapies particularly for tumors, which are noncurative by conventional means. The role of IL-13Rα2 in thyroid cancer cell lines and the safety and efficacy of IL-13PE for thyroid cancer therapy is currently being studied in animal models.

Enhancement of Antitumor Activity Using Cetuximab and Novel Epidermal Growth Factor Receptor (EGFR) Peptide-specific T Cells

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Therapeutic targeting of the epidermal growth factor receptor (EGFR), which is highly overexpressed and correlated with poor prognosis in colon and head and neck squamous cell carcinoma (HNSCC), has shown clinical efficacy using blocking mAbs, cetuximab or panitumumab, but only in 10% to 20% of patients. Clinical responsiveness is not correlated with level of EGFR expression by tumor cells, but is correlated with certain Fcγ receptor (FcγR) genotypes, suggesting immune activity other than inhibition of ligand binding may be responsible for therapeutic efficacy. Cetuximab-resistant tumor cells demonstrate ubiquitination and degradation of EGFR, the first step in antigen processing, which would increase its potential as a target for cytotoxic T lymphocytes (CTLs). Thus, T-cell–based immunotherapy may enhance the antitumor efficacy of EGFR-specific mAb. We identified a novel immunogenic wild-type EGFR CTL defined peptide and modified its sequence to enhance HLA-A*0201 binding and stimulation of anti-wt EGFR peptide-specific CTL. We found that these EGFR-specific CTLs recognized colon and HNSCC cells in a peptide-specific and HLA-A*0201 restricted fashion. Incubation of HNSCC cells with cetuximab or panitumumab led to enhanced EGFR internalization and presentation to EGFR-specific CTL. Thus, a novel immunogenic EGFR peptide-specific CTL epitope may be useful for potential combinatorial immunotherapy with EGFR-specific mAbs in cancer patients.

Interleukin 13 (IL-13) Mediates Signaling Through IL-13Rα2: Targeting of the Receptors by IL-13 Pseudomonas Exotoxin (IL-13PE) for Pancreatic Cancer Therapy

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Despite modern medical advances, there are limited therapeutic options for pancreatic cancer therapy and it still is the major cause of high morbidity and mortality. We have reported that interleukin-13 pseudomonas exotoxin (IL-13PE) cytotoxin is highly cytotoxic to tumors expressing IL-13Rα2 chain. In the present study, we report that 37 of 70 (52%) tumor samples from patients with pancreatic cancer express moderate-density to high-density surface IL-13Rα2 by an immunohistochemical assay, whereas normal pancreatic samples (n = 17) expressed no or low-density IL-13Rα2. In addition, we demonstrate that IL-13Rα2 is involved in induction of transforming growth factor-β1 promoter activity when stimulated by IL-13 in IL-13Rα2-positive pancreatic cancer cell lines but not in IL-13Rα2-negative cell lines. The c-jun and c-Fos nuclear transcription factors of the AP-1 family were found to be activated by IL-13 in these cell lines, whereas no activation was observed in IL-13Rα2-negative cell lines. IL-13PE cytotoxin was highly cytotoxic to 3 IL-13Rα2-positive pancreatic cancer cell lines with IC50 ranging from < 0.1 to 12 ng/mL, whereas no cytotoxicity was observed in normal human pancreatic ductal epithelium cells, and human umbilical vein endothelial cells even at concentrations $> 1{,}000$ ng/mL. IL-13PE cytotoxin administered to mice with metastatic and orthotopic tumors caused a significant decrease in tumor volume and prolonged the survival of mice in both early cancer and advanced metastasis model. Continuous intraperitoneal infusion of IL-13PE cytotoxin using mini-osmotic pumps was more effective in regressing the tumor volume than bolus intraperitoneal injections. Thus, our results indicate that IL-13Rα2 plays a key role in pancreatic cancer and that IL-13PE may require further consideration as a therapeutic agent for the treatment of pancreatic cancer.

Regulatory T Cells in Mouse Models of Human Prostate Cancer

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Regulatory T cells (Tregs) are thought to be responsible for suppressing the immune response induced by immunotherapeutic vaccination against prostate tumor-associated antigens, thereby limiting the efficacy of this novel treatment strategy. Very recently, we showed that therapeutic vaccination of TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) mice can control prostate tumor outgrowth and elicit very long-term survival. This effect was observed when mice were vaccinated at 8 weeks of age when they have precancerous prostatic intraepithelial neoplasia lesions, but not at later stages of tumorigenesis. Clinical trials of prostate cancer immunotherapy typically involve men with very advanced disease, and have generally been disappointing. We hypothesize that Tregs accumulate in the prostate tumor over time, and can suppress the immune response to vaccination when a critical threshold is crossed. The number of suppressive T-cell subsets has grown rapidly. To develop strategies to attenuate their activity in vivo, it is critical to identify which Treg subpopulations are present within the prostate tumor at each stage of disease progression. To this end, we used 2 models of human prostate cancer, the TRAMP mouse and the new prostate-specific conditional PTEN knockout (CPP) mouse. Most TRAMP mice develop neuroendocrine tumors, while the majority of CPP mice develop adenocarcinomas. We recently identified CD8+ CD25+Foxp3+ Tregs within the prostate tumors of 12-week-old TRAMP mice. To identify other Treg populations, prostate tumor-infiltrating lymphocytes were isolated from young (12 to 16 wk) and old (33 to 42 wk) TRAMP and CPP mice and analyzed by flow cytometry. In young TRAMP mice, 3 Treg subpopulations, CD4+Foxp3+ T cells, CD8+Foxp3+, and CD3+CD4−CD8−NK1.1− double negative T cells, were identified. In aged TRAMP mice, only CD4+Foxp3+ T cells and CD3+CD4−CD8−NK1.1− double negative T cells were observed. A dramatic increase in the number of CD4+Foxp3+ Tregs per gram of prostate tumor was observed in aged TRAMP mice compared to younger animals. In contrast to TRAMP mice, only CD4+ Foxp3+ Tregs were identified in the prostate tumors of young CPP mice. In the spleens of older CPP mice, both CD4+Foxp3+ and CD8+Foxp3+ were observed. In addition, CD4+CD25+Foxp3+ Tregs isolated from the tumor-draining lymph nodes of these mice strongly suppressed T-cell proliferation. Whether Tregs accumulate in the tumors of older CPP mice remains to be determined. Our results show that at least 3 Treg subsets can be identified in mouse prostate tumors. The number and type of Tregs that accumulate are dependent on the type of prostate tumor and its stage of development. These data allow mechanistic studies of Treg activity in prostate tumors, and intervention in order to enhance therapeutic vaccine efficacy.

Cancer Vaccines Regulate the Lymphoid/Memory Versus Peripheral/Effector Pathway of Differentiation of CD8$^+$ T Cells

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Effective CD8$^+$ T-cell responses require participation of both the CCR7$^-$/CCR5$^+$ effector [cytotoxic T lymphocytes (CTLs)] and CCR7$^+$/CCR5$^-$ memory cells with respective peripheral versus lymphoid homing properties. Here, we report that CD8$^+$ T cells activated by the "inflammatory-type" dendritic cells (DCs) activated by interferons and or Toll-like receptor ligands (αDC1s; type-1 polarized DCs), expand and acquire interleukin (IL)-7R and IL-15 R, but also develop CTL functions and undergo a switch in chemokine responsiveness (from lymphoid to peripheral). In contrast, CD8$^+$ T cells activated by "exhausted" long-term–stimulated DCs or by DCs exposed to mediators of chronic inflammation, such as prostaglandin E_2 (PGE_2), effectively expand and acquire IL-7R$^+$/IL-15R$^+$ phenotype while retaining the CD62L$^+$CCR7$^+$ CCR5$^-$ central memory phenotype not associated with CTL functions. The "noncytolytic/lymphoid" status induced in naive or memory (eg, tumor-specific) CD8$^+$ T cells by the PGE_2-matured DCs could be fully reversed by the αDC1s, suggesting that the noncytolytic T cells are fully functional but undergo memory-type differentiation.

The independent regulation of CD8$^+$ T-cell expansion and their commitment to the peripheral/effector versus lymphoid/memory subsets helps to understand the preferential induction of memory CD8$^+$ T cells at late stages of immune responses and the negative impact of persistent inflammation on the development of immune memory. The limited effectiveness of PGE_2-matured DCs in inducing tumor-relevant chemokine receptors (CCR5 and CXCR3) helps to explain the limited clinical effectiveness of currently used DC-based cancer vaccines, facilitating the development of effective forms of immunotherapy.

Intra-lymphatic Continuous Infusion of Dendritic Cells in Patients With Advanced Melanoma: Early Indication of Clinical Efficacy

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Therapeutic cancer vaccines need to function in the presence of suppressive T*regs* and CD8$^+$ cytotoxic T lymphocytes (CTLs) present in peripheral tissues of cancer patients. In order to assure rapid delivery of "non-exhausted" dendritic cells (DCs) to the lymph nodes and to avoid their inactivation/elimination by peripheral Tregs and CTLs, we have developed a semicontinuous intralymphatic mode of vaccine delivery, using implantable lymphatic cannulas. This approach allows the efficient and rapid delivery of vaccines to draining lymph nodes without disruption of the nodal structure. It also allows for repeated/semicontinuous delivery of vaccines over prolonged time periods, mimicking the kinetics of the migration and persistence of functional DCs during physiologic immune responses.

We have completed the initial safety evaluation of standard DC-based and αDC1-based semicontinuous intralymphatic vaccines (25,000 DC per injection; 12 injections over 4 days; a total of 300,000 DCs) in 6 patients with stage IIIb-IV melanoma in trial UPCI 03-118 and are now proceeding to the comparative evaluation of "high" doses (250,000 DC per injection) in additional 14 patients. Four of the initial 6 patients successfully completed the protocol and received 2 courses of the intralymphatic DC infusions. Two patients dropped out before the second course of treatment for the reasons unrelated to the protocol. Prolonged lymphatic cannulations and semicontinuous DC delivery were feasible and safe.

Unexpectedly, already at this ultra-low dose-level (10-fold to 100-fold fewer DCs than routinely used doses of intradermal or intranodal vaccines) we have observed evidence of clinical efficacy of vaccination in 3 of the 6 patients, including 3 of the 4 patients who received both courses of vaccination.

Among the 4 patients who successfully completed the protocol, progressive disease was observed only in 1 patient (stage IV). In the remaining 3 patients, we observed 1 stabilization of stage IV disease (lung; >6 mo long), and 2 partial antitumor responses in patients with stage IIIb disease (1 near-complete and ongoing for >12 months; another ongoing for >2 mo).

The current data demonstrate the feasibility of prolonged intralymphatic delivery of biotherapeutic agents in patients with advanced cancer and provide preliminary indication that DC-based cancer vaccines can be clinically effective at ultra-low doses, up to 100-fold lower than the doses currently considered as necessary.

Enhanced Depot Vaccine Formulations for Targeted Peptide-based Cancer Immunotherapy

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Parenteral peptide-based vaccination strategies have been largely ineffective in clinical trials due to many factors including antigen selection, the limited immunogenicity of short peptides, and the inability to overcome cancer-induced immune suppression. A common approach today is to formulate peptides in oil-based emulsions such as Montanide ISA51 in combination with granulocyte macrophage-colony stimulating factor adjuvant to induce a peptide-specific immune response. We have developed depot vaccine formulations with enhanced immune activating potential, which in combination with carefully selected peptide antigens, may help overcome the limitations of current peptide-based vaccination strategies.

Naturally occurring peptides (IMT peptides) bound to HLA class I molecules of cancer cells have been previously identified by a mass spectrometry method and their recognition by HLA-restricted cytotoxic T lymphocytes (CTLs) has been confirmed. A delivery platform that is based on an enhanced depot formulation of antigens and a proprietary adjuvant has been developed and tested in an established pre-clinical challenge model; an established CTL epitope from HPV16 E7 formulated in the delivery technology, named DepoVax, effectively eliminated C3 tumors in a therapeutic challenge model with a single dose.

Antigen-specific CTL activity was detected against non-IMT peptide loaded breast, ovarian, and prostate cancer cell lines indicating that all IMT peptides are naturally processed and presented by these cells. In contrast, no CTL activity could be detected against cells from normal tissues, suggesting differential processing of these tumor-associated antigens in tumor cells. A DepoVax-based vaccine containing a mixture of these HLA-A2 restricted epitopes potentiated an immune response in HLA-A2 transgenic mice. DepoVax-based formulations induced lower levels of CD4+CD25+Foxp3+ regulatory T cells compared to control vaccines formulated with Montanide ISA51.

This multi-targeting strategy using peptides derived from antigens involved in maintaining the malignant phenotype, in combination with a potent vaccine delivery technology, is believed to induce an effective immune response while minimizing tumor escape. Preparations for a clinical trial are underway.

Immunization of a Recombinant Plasmid Encoding Hepatitis C Virus Core Protein

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DNA vaccines express antigens intracellularly and effectively induce cellular immune responses. The immunogenicity and protective efficacy of DNA vaccines have been demonstrated in numerous animal models of infectious diseases. In order to increase the potency of DNA vaccines, in this study, conventional adjuvants such as aluminum phosphates, dendrosome, CpG motif, and mixture of aluminum phosphate and CpG motif have been tested. Female BALB/c mice were immunized with mixture of 10, 25, and 50 μg hepatitis C virus (HCV) core pcDNA3. Each recombinant pcDNA3 together with different adjuvants used as an immunogenic vaccine were injected 3 times on day 0, and 30 and 50 days. Blood samples were collected at 4 different times intervals and antibody response against HCV core antigen was determined by HCV core enzyme-linked immunosorbent assay kit.

The results indicate that the best antibody response was with mixture of aluminum phosphate and CpG motif as an adjuvant. This data suggest that the antibody response induced following DNA immunization can be modified by formulation strategies (Fig. 1).

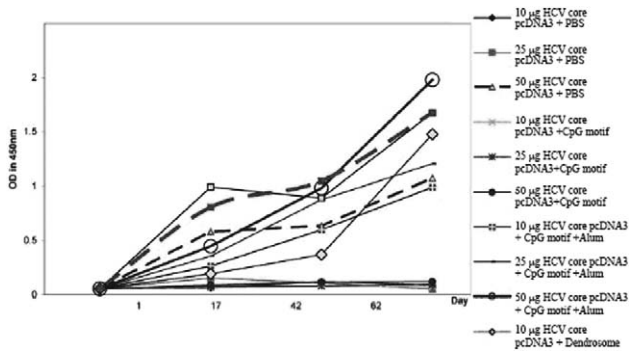

FIGURE 1.

Development of an Effective Cancer Immune Therapy by Cbl-b Silencing

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Anticancer immunotherapy is an increasingly attractive approach to employ the immune system to specifically eliminate tumors. However, the effectiveness of such therapies is often limited, which has been attributed to the abilities of established tumors to modulate the host immune system, particularly by generating an immunosuppressive cytokine milieu, impeding T-cell costimulation signaling pathways and induction of T regulatory cells.

The E3 ubiquitin ligase cbl-b has been identified as an important gatekeeper controlling T-cell activation and therefore maintaining the balance between tolerance, activation, and autoimmunity. Cbl-b is an intrinsic mediator of T-cell anergy that defines the activation threshold of the antigen receptor and negatively regulates CD28 costimulation. In addition, cbl-b is also essential for the effects of transforming growth factor-β on induction of T regulatory cells and cbl-b deficient effector T cells are resistant to suppression by T regulatory cells. Thus, cbl-b is a natural target to enhance antitumor immune activity.

Concordantly, cbl-b deficient mice are protected against tumor development. Moreover, transfer of cbl-b deficient CD8 T cells into mice bearing already established tumors is sufficient for tumor eradication. This proof of concept in a murine model shows the route to a new cancer immune therapy protocol, based on the transient ex vivo inhibition of cbl-b in patient T cells directed against tumor antigens, which are thereafter transferred back to the patient by adoptive cell therapy.

To this end, we have investigated the role of cbl-b in the context of human T-cell responses. We show here the kinetics of cbl-b regulation in human CD4 and CD8 T cells and thus define the entry points to interfere with cbl-b functions. We have identified siRNAs, which were able to shut down cbl-b expression following transfection into primary human T cells resulting in almost complete ablation of cbl-b protein in siRNA-treated cells. The consequences of cbl-b silencing in human T cells were in agreement with those reported for murine T cells, particularly the secretion of key cytokines for T-cell activity and tumor rejection, like interleukin-2 and interferon-γ, was enhanced thereby providing a first proof of this concept. Based on these results, we will present a protocol for silencing cbl-b in human T cells to enhance antitumor immune responses.

Identification of MHC Class II Restricted Epitopes in the Tumor Associated Antigen BCL-X(L)

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Introduction: The Bcl-2 family comprise several well-characterized tumor-associated antigens [ie, Bcl-2, Bcl-X(L) and Mcl-1]. Spontaneous CD8+ T-cell responses against these proteins have been identified as frequent features in cancer patients. To address the potency of these proteins as vaccination targets, clinical trials in patients suffering from multiple myeloma are currently set to begin October 2008 at University Hospital Herlev, Denmark. In an effort to enhance the vaccines, we would like to include MHC class II epitopes in future clinical trials. We therefore aim at characterizing MHC class II restricted peptides in the anti-apoptotic protein Bcl-X(L).

Materials and Methods: Twenty to 23 amino acid overlapping peptides spanning the Bcl-X(L) protein were synthesized. Melanoma patient peripheral blood lymphocytes were cultured for 7 days with autologous dendritic cells pulsed with the different peptides and screened for release of interferon-γ in ELISPOT assays.

Results: Nine out of 10 patients exhibit responses against at least 1 of the 9 peptides that are included in the study. One response was repeated and by cell sorting shown to be a CD4+ T-cell response. Responses were not detectable in direct ELISPOT assays without the prestimulation with peptide-pulsed dendritic cell.

Discussion: Various class II epitopes from the Bcl-2 family protein Bcl-X(L) were identified and were shown to elicit spontaneous immune responses in cancer patients suffering from malignant melanoma. After further validation, the best candidate peptides will be included in future Bcl-X(L)-based clinical vaccination trials at University Hospital Herlev.

Different Quality of T Cell Responses to WT1 Peptide Vaccination in Patients With AML/MDS and Patients With Solid Tumors

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Introduction: The transcription factor Wilms tumor protein (WT) 1 holds great promise for cancer immunotherapy, which is expressed in many hematologic and solid malignancies, and is essential for tumor-cell proliferation. We have initiated 2 clinical vaccination trials, 1 for patients with WT1-expressing acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and another for WT1 expressing carcinomas. The study in AML/MDS proved good immunological efficacy of vaccination with the HLA-A2–restricted WT1.126-134 peptide administered with adjuvants granulocyte macrophage-colony stimulating factor and T helper protein keyhole limpet hemocyanin. To compare the immunogenicity between patients with AML/MDS and carcinoma we analyzed the T-cell response in peripheral blood (PB).

Patients and Methods: Twenty-four AML patients and 2 MDS patients as well as 9 patients with ovarian cancer (n = 6), thyroid cancer (n = 2) and breast cancer (n = 1) were accrued in both studies, of which to date 25 AML/MDS and 8 carcinoma patients were evaluable for immune response. Clinical efficacy was observed in both trials in a fraction of patients. Quantitation of the T-cell response to the WT1.126-134 epitope was performed in PB before, during, and after vaccination by tetramer staining.

Results: Whereas 28% of the AML/MDS patients had a spontaneous WT1-tetramer response, none of the 9 cancer patients had a WT1–T-cell response at baseline. After 2 to 4 vaccinations WT1-specific T-cell responses were detectable in 54% of AML/MDS patients and 67% of cancer patients, resulting in an early conversion rate of 26% in AML/MDS and of 67% in carcinoma patients. After 6 vaccinations, WT1-specific T-cell responses were present in 78% of AML/MDS and in 40% of carcinoma patients. Thus, the overall conversion rate was 56% in AML/MDS and 75% in cancer patients. In contrast to the AML/MDS patients in which the PB T-cell responses remained detectable at weeks 10/18 in most patients, all WT1-specific CD8+ T-cell responses in PB of cancer patients were transient despite of continuation of vaccination and ongoing clinical efficacy.

Conclusions: These results demonstrate profound differences in spontaneous and vaccine induced T-cell response among patients with hematological and solid malignancies, suggesting considerably higher spontaneous immunogenicity of AML/MDS, a higher conversion rate in carcinoma patients compared to AML/MDS and in contrast to AML/

MDS WT1-specific T-cell responses in the PB of carcinoma patients which were only transient, probably due to migration into the tumor. Detailed analyses of T-cell activation patterns, migratory capacity, and regulatory effects are currently being performed.

Low Lymphocyte and Neutrophil Counts at Start of Vaccinations With the Cancer Vaccine IGN101 Predict Improved Relapse-free Survival-results of a Randomized Phase II/III Trial in Adjuvant NSCLC

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IGN101 is a cancer vaccine acting via the epithelial cell adhesion molecule (EpCAM) expressed on epithelial cancers. It consists of 0.5 mg alum-adsorbed immunogenic murine Mab 17-1A that bears EpCAM mimotopes to trigger an immune response against EpCAM by repeated subcutaneous injections. As cancer vaccines may work best in the adjuvant setting, a placebo-controlled phase II/III study was performed in adjuvant nonsmall cell lung cancer (NSCLC) patients.

Between December 2001 and April 2005, 762 NSCLC patients (pts) were randomly assigned in 26 European centers to receive IGN101 (382 pts) or placebo (380 pts). Placebo consisted of aluminum hydroxide suspension without protein. Within 1 to 3 weeks after R0 resection in stage Ib-IIIa, treatment was applied subcutaneously on days 1, 15, 29, 71, and then every 3 months up to 3 years, and every 6 months thereafter. No other preoperative or postoperative therapy was permitted except postoperative radiotherapy in stage IIIaN2. Data lock was in April 2007, two years after accrual of the last pt. Primary end point was relapse-free survival (RFS), secondary end points were tolerability, immunogenicity, overall survival, and quality of life. As in an earlier placebo controlled trial with IGN101 and based on an exploratory analysis, pts with low lymphocyte and low neutrophil counts at entry had improved survival when treated with IGN101 (iSBTc 2007), for this NSCLC trial a prospectively defined subgroup analysis was performed for pts presenting with low neutrophil and low lymphocyte counts at entry.

Based on intention-to-treat, there was no difference in RFS (median IGN101 819 d, placebo 736 d; $P = 0.793$). However, pts with lower neutrophil counts at entry (threshold 5×10^9/L) showed a trend to improved RFS when treated with IGN101 (median 1096 vs. 726 d; HR = 0.735, $P = 0.196$). For those pts presenting both with lower neutrophil and lower lymphocyte counts at entry (thresholds 5×10^9/L and 1.6×10^9/L, respectively) a significantly longer RFS was found (median IGN101 not reached, placebo 459 d; HR = 0.482, $P = 0.034$). Vaccinations were very well tolerated.

In conclusion and shown by prospectively defined subgroup analysis, NSCLC pts stage Ib, II, and IIIa vaccinated with the cancer vaccine IGN101 following R0 resection have improved RFS when neutrophil and lymphocyte counts at entry are below 5×10^9/L and 1.6×10^9/L, respectively. A further randomized trial with IGN101 using above predictive parameters as inclusion criteria is warranted to finally confirm these results.

Clinical Response to the MAGE-A3 Immunotherapeutic in Metastatic Melanoma Patients is Associated With a Specific Gene Expression Profile Present at the Tumor Site

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Background: This study was designed to select the optimal combination of MAGE-A3 recombinant protein with an Adjuvant System (EORTC 16032-18031/NCT00086866). In addition, gene expression profiling was used to identify markers predictive of the clinical activity of the MAGE-A3 ASCI recorded in this phase II study.

Methods: 75 patients (pts) with progressive, unresectable stage III or stage IV M1a MAGE-A3 (+) melanomas, were randomized as first-line therapy between immunization with MAGE-A3 protein and Adjuvant Systems AS15 or AS02B (GSK proprietary). Gene expression profiling (Affymetrix) was performed on tumor biopsies taken preimmunization.

Results: Four objective responses (OR) were reported in the AS15 arm versus 1 OR in the AS02B arm. Stable disease (SD) ≥ 16 weeks was reported in 5 pts in each arm and several mixed response (MxR) were reported in each arm

Initial analysis using supervised hierarchical clustering of 2 OR with 7 nonresponders identified 2 gene clusters based on differential expression. The correlation of this gene expression signature (GS) and OR was further confirmed on 22 pts. MxR and SD clustered with OR, suggesting a strong association between the identified signature and the MAGE-A3–induced clinical benefit. Independent validation on additional 30 pts confirmed the association of clinical benefit and GS. Most of the identified genes are immune-related, defining a particular biological context in the tumor environment before immunization. The signature was randomly distributed in the 2 study arms and identified all pts with clinical benefit (OR, MxR, SD). Selection of pts with the GS results in increased clinical efficiency as illustrated by the median time to treatment failure: 2.3 months in the GS (−) and 10.3 months in the GS (+) population (HR = 0.31; 95% confidence interval, 0.13-0.76).

Conclusions: The AS15 arm has shown to induce a higher MAGE-A3 immune response and more frequent clinical activity. The gene expression in metastatic melanoma is strongly correlated with clinical activity to the MAGE-A3 ASCI treatment. This signature reflects an immune microenvironment in the tumor prior to MAGE-A3 ASCI treatment. Interestingly, this signature has also been independently reported to be correlated with clinical activity to MAGE-A3 treatment in a randomized double-blind phase II study in resected NSCLC. In this setting, increased activity is also reported in the enriched populations. This predictive gene signature will be prospectively validated in future phase III trials.

Development of a Novel Multiplex Cancer Vaccine for the Therapy of Patients With Melanoma

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Background: Cancer immunotherapy remains a promising, albeit elusive, therapeutic approach for melanoma and other cancers. Likewise, active immune therapy has resulted in mixed results in immune response assays (ie, enzyme-linked immunosorbent spot, increases in antibody titers to tumor antigens, and various cytotoxicity assays) with an apparent lack of association between immune response and clinical outcome. Recently, dendritic cell (DC)-based vaccination to enhance antigen presentation to the immune system has provided promising preliminary results in clinical trials, including melanoma.

Objectives: The main objective of this study was to develop a novel multiplex therapeutic cancer vaccine (PMTCV) designed for efficient activation and uptake of clinically relevant peptide epitopes by DC with the aim of generating a potent and durable antitumor response.

Methods: Oxy-amino derivatized quenched fluorescent-labeled peptides with cleavable sequences (cathepsin D) were ligated to an amino-polyvinyl alcohol (PVA) backbone with methyl ketone moieties. In vitro studies (enzymatic and cellular) were conducted to demonstrate the feasibility of cleaving peptides off the PVA scaffold.

Results: We have constructed a PMTCV using fluorescent-labeled based peptides, in order to test the feasibility of enzymatically cleaving peptide epitopes from the scaffold. We have demonstrated partial loading of peptide onto the scaffold using methyl ketone conjugation. The synthesis and conjugation is currently being optimized. We have demonstrated cathepsins are capable of selectively cleaving the linkers, an important step in releasing the peptides for subsequent loading of DC. Further immunologic studies (lymphoproliferation and Pmel-1 model animal studies to detect antitumor responses) with a newly synthesized PMTCV ligating poly (I:C) and Pmel-1 peptide to the PVA scaffold are planned.

Conclusions: The reported successful construction of the PMTCV is the first step in the generation of a therapeutic vaccine that has the potential of generating a more potent host immune response than traditional approaches. The PMTCV can be synthesized easily and tailored to include known peptide epitopes of different tumors. The vaccine has the potential to provide long-lasting cytotoxic T lymphocyte and T_H1 activation. Such an approach has the real possibility of providing meaningful clinical benefit in the relatively near future.

Induction of Anti-Tumor Activity in HER-2 Tolerant Mice and of Antigen Spreading by MVA-BN-HER2

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MVA-BN-HER2 is a MVA-BN–derived, replication-deficient vector designed for the treatment of breast cancer that is currently being evaluated in phase I clinical trials. We have previously shown that MVA-BN-HER2 is a potent immunogen, which induces antigen-specific humoral and cellular responses and shows therapeutic activity in several mouse tumor models. Here, we present evidence that further emphasizes the potency of MVA-BN-HER2 as a cancer vaccine candidate with the demonstration of its ability to (1) break tolerance in HER-2 transgenic mice and (2) mediate antigen spreading.

Studies were performed to compare the immune responses in HER-2–tolerant WAP and C57BL/6 wild-type mice and, although dampened in HER-2–tolerant mice, HER-2–specific antibody and T-cell responses were elicited in both mouse strains. Moreover, HER-2 transgenic mice (30%) could sustain a lethal challenge with syngeneic MC38-HER-2 cells when treated with MVA-BN-HER2. In separate experiments, we examined whether MVA-BN-HER2 activity could result in the induction of immune responses against MC38 tumor-derived antigens other than HER-2. We found that mice that rejected a lethal challenge with MC38-HER-2 were also protected form subsequent challenge with the parental MC38 cells. Overall, our data indicate that MVA-BN-HER2 can overcome tolerance in HER-2 transgenic mice and that MVA-BN-HER2–mediated tumor therapy resulted in the induction of protective immune responses to non–HER-2 tumor antigens. Breaking of tolerance and antigen spreading are critical cancer vaccine properties. Indeed, HER-2 is a tumor-associated "self"-antigen that is typically unmodified in cancer and anti–HER-2 immune responses can be hampered by peripheral tolerance. Also, antigen spreading may overcome the challenges of treating heterogeneous tumors and the possible selection of HER-2–negative variants in humans. Hence, these findings further demonstrate that MVA-BN-HER2 is a potent vector for cancer immunotherapy.

Post-apoptotic Tumors are Efficiently Engulfed by Dendritic Cells and Enhance Their Antigen Cross-Presentation Activity

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The context in which tumor-associated antigens are delivered to dendritic cells (DCs) influence the DC cross-presentation efficiency. Drug-induced apoptosis has recently been implicated in the in vivo cytotoxic T lymphocyte cross-priming. In this situation, however, dying tumor cells may exceed the DC default apoptotic clearance program and proceed to secondary necrosis, thus changing their molecular pattern.

Here we have addressed the relevance of postapoptotic death on renal carcinoma cells (RCC) by using different death stimuli. UVC, but not γ-irradiation or anthracyclins, induced after 4-hour treatment of the RCC cell line K1, a combination of apoptotic (phosphatidylserine and calreticulin plasma membrane mobilization) and necrotic (membrane incompetence) features, a pattern referred to as secondary necrosis.

Heat shock protein (Hsp)-70 and chromatin-bound high mobility box 1, HMGB1 protein, typical of necrosis were released during the further 20 hours and thus made accessible to cocultured monocyte-derived immature DC. UVC-treated, secondary necrotic RCC cell lines were cross-presented with higher efficiency by cytokine-matured DC than their early apoptotic (ie, γ-irradiated) counterpart.

Upstream events such as (a) tumor uptake, (b) activation of genes involved in the antigen processing machinery (ie, TAP and Type I interferon), (c) expression of costimulatory (CD80) and maturation (CD83 and CCR7) molecules were also up-modulated after loading immature DC with secondary necrotic, but not apoptotic, tumor cells.

These data offer a description of the molecular and immunogenic characteristics of postapoptotic tumors, which can explain the immunogenicity of tumor cell death induced by chemo/radiotherapy and be exploited to increase the efficiency of ex-vivo tumor-associated antigen delivery to the DC cross-presentation pathway.

Treatment of Late Stage Cancer Patients With Cyclophosphamide in Combination With ZYC300 Results in a Decrease in Treg Mediated Immuno-suppression and an Increase in T-cell Immune Responses to CYP1B1

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A phase I clinical trial was conducted to test the safety of a novel immunotherapeutic compound, ZYC300. ZYC300 is a cancer immunotherapeutic consisting of plasmid DNA (pDNA), which encodes cytochrome P450 1B1 (CYP1B1) encapsulated in PLG microparticles. CYP1B1 has been shown to be overexpressed in many tumor types with a relatively restricted normal tissue expression profile. ZYC300 was tested in combination with cyclophosphamide pre-treatment in an effort to reduce the number of immunosuppressive regulatory T cells (Tregs), while priming and expanding an effector T-cell response specific to CYP1B1 peptides.

Twenty-two late stage cancer patients were enrolled in the clinical study. The cancer types included breast, colorectal, ovarian, renal cell, and prostate. Patients were given an intravenous dose of 600 mg/m^2 cyclophosphamide 3 days prior to an intramuscular injection of 400 mcg ZYC300. This cycle of treatment was repeated every 2 weeks for up to a total of 6 treatment cycles. While safety was the primary end point, in addition, clinical and immunological responses were measured. Preliminary results of this trial were recently presented at the 2008 annual meeting for the American Association of Cancer Research.[1]

In order to assess the immune status of the patients on this trial, peripheral blood mononuclear cells were isolated from venous blood or leukophoresis samples from the patients prior to and during the trial. Two functional assays were used to measure Treg activity in the patients including natural killer cell and T-cell proliferation assays. The interferon-γ ELISPOT assay was performed to measure CD3+ T-cell responses to pools of overlapping CYP1B1 peptides.

Of the 11 patients who received all the 6 treatment cycles, Treg activity was reduced in 6 patients as measured by the functional assays. There was good correlation between these 2 assays. Twenty of 22 patients were tested for CYP1B1 immunity. Twelve of 20 patients had CD3+ T-cell responses that were elevated above their baseline levels as assessed by the interferon-γ ELISPOT assay. Interestingly, 3 patients had measurable antitumor activity as measured by RECIST criteria, tumor markers, and/or liver enzymes. These 3 patients also had the highest levels of CYP1B1 CD3+ T-cell responses. Taken together, these data suggest an immunological effect in response to the dosing regimen (cyclophosphamide followed by ZYC300).

Reference:

1. Nadler LM, et al. Anti-CYP1B1 biologic and clinical responses are induced in patients with advanced stage solid tumors with cyclophosphamide pre-dosing and immunization with ZYC300. *AACR*. 2008.

Phase I Clinical Results Comparing Local and Systemic Administration of an APC-targeted Cancer Vaccine

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Efficient antigen delivery to antigen presenting cells (APCs) is a critical component of effective in vivo immunization strategies. Antigens attached to B11 (a human antibody against the mannose receptors expressed on interstitial dendritic cells and macrophages) have been shown to be processed and presented much more efficiently than nontargeted antigens, and generate robust immune responses when combined with toll-like receptor (TLR) agonists. Importantly, this targeted vaccination approach allows potential access to a larger APC population compared to standard protein vaccination strategies. CDX-1307 is a vaccine composed of B11 fused with the β subunit of human chorionic gonadotropin (hCG-β), a tumor-associated antigen that has been correlated with advanced stage of disease and poor prognosis. Two phase I studies were designed to compare different routes of administration, intradermal (ID) delivery versus systemic (IV) of CDX-1307. Both studies used 4 biweekly doses of the vaccine and enrolled patients with advanced epithelial cancers known to frequently express hCG-β. The ID study dose escalation is complete, with 25 patients treated with CDX-1307 at doses of 0.3, 1.0, 2.5 mg, and 2.5 mg plus granulocyte macrophage-colony stimulating factor (GM-CSF). In the IV study, 15 patients have been treated with CDX-1307 at doses of 1, 3, 10 mg, and 10 mg plus GM-CSF, and accrual into the final cohort with 30 mg ± GM-CSF is ongoing. Dosing with CDX-1307 alone or in combination with GM-CSF has been well tolerated to date. Treatment-related toxicities were generally mild to moderate-mild injection site reactions and fatigue in the ID study, and fatigue, myalgia and flulike illness in the IV study. An increased rate of injection site reaction was seen in the groups that received GM-CSF; otherwise, the safety profile was relatively unchanged from the groups to receive CDX-1307 alone. The CDX-1307 was shown to be localized in dermal macrophages and DCs in posttreatment biopsies following intradermal administration of CDX-1307. Analysis of humoral immune responses to hCG-β were detected in both ID and IV study, and GM-CSF appeared to enhance the responses. Two patients in the IV study (at 10 mg and 10 mg plus GM-CSF) experienced stable disease for at least 6 months. One patient with pancreatic cancer experienced a significant mixed response. Near completing dose-escalation studies, we are now initiating combination studies with the TLR3 agonist Poly-ICLC (Hiltonol) and TLR7/8 agonist (resiquimod). These combinations are expected to maximize the potential for the targeted vaccine strategy.

Enhancing Immunogenicity of Therapeutic Vaccines in the Immunotherapy of Ovarian Cancer

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Background: We recently completed 2 clinical studies of oregovomab for advanced ovarian cancer. One phase II study was in the front line chemo-immunotherapy setting and the other study was postfront line maintenance mono-immunotherapy (Berek 2008). Paradoxically, the combination chemo-immunotherapy setting produced enhanced immune induction as assayed by humoral response to administered antibody and T-cell response to CA125 (the antibody target) by enzyme-linked immunosorbent spot. Furthermore, the 40-patient combination chemo-immunotherapy study, identified an additional significant effect of schedule on immune response for combination carboplatin paclitaxel therapy. The simultaneous (over 1 wk delayed) immunization strategy gave the most vigorous bioactivity (Braly 2007). Forty-four percent of vaccinated patients generated a treatment emergent T-cell response to CA125 with the simultaneous chemo-immunotherapy schedule, whereas 22% of 1-week delayed patients and only 5% to 10% of those in the maintenance setting mounted such responses.

Objective: The current study aimed to identify a clinically practical approach to further enhance the immune response achievable with oregovomab by the addition of an immune adjuvant.

Methods and Results: In considering possible adjuvants for clinical use, we identified that polyI:polyC12U (Ampligen) is a clinical grade reagent that is currently under NDA review for approval in the treatment of chronic fatigue syndrome (Hemispherx Biopharma, Philadelphia). The modified dsRNA molecule is a specific Toll-like receptor-3 (TLR3) agonist with a large safety experience from parenteral administration in recent clinical trials. TLR3 is a MyD88 independent signaling pathway, and therefore TLR3-specific agents are not redundant to other TLR ligands. We tested the agent in our human peripheral blood mononuclear cell dendritic cell (DC) antigen processing culture system. Maturation markers were assayed using flow cytometry, cytokines measured using a Multiplex assay, specific T-cell responses were assayed by ICC flow cytometry. We found, at currently used therapeutic serum concentrations of polyI:polyC12U, effective DC maturation and local cytokine release including interleukin-6, interleukin-12, tumor necrosis factor-α, interferon-γ, and the chemokines MIP1α and MCP1. The agent greatly enhanced the T-cell response to a panel of recall antigens, and when used in an in vitro DC antigen-processing assay in combination with oregovomab and CA125, significantly potentiated interferon-γ–positive CD4 and CD8 T-cell responses to tumor antigen. Additional immunization protocols are ongoing.

Conclusions: PolyI:polyC12U appears useful as a systemic cancer vaccine immune potentiator. Clinical study of this agent with oregovomab and in combination with other cancer immunotherapeutics is justified. The addition of this TLR3 agonist is a promising strategy to further amplify antitumor specific immunity.

Induction of Type-1 CTL Responses Against Novel Glioma-associated Antigen (GAA)-Derived Epitopes EPHA2 (883 to 891) and IL-13Ralpha2 (345 to 353) in Patients With Recurrent Malignant Glioma Receiving Type-1 Dendritic Cell (DC) Vaccines in Combination With Poly-ICLC

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Our previous preclinical studies have demonstrated that intramuscular administration of a Toll-like receptor 3-ligand poly-ICLC remarkably enhances induction of type-1 cytotoxic T-lymphocytes (CTLs) and improves therapeutic efficacy of vaccinations against glioma-associated antigen-derived CD8+ T-cell epitopes. Based on these studies, we have developed a phase I/II trial. Human leukocyte antigen (HLA)-A2+ participants with recurrent malignant glioma received 4 intralymph node injections of 1×10^7 type-1 dendritic cells (DCs) loaded with HLA-A2 binding peptides EphA2 (883 to 891), interleukin (IL)-13Rα2 (345-353:1A9V), YKL-40 (202-211), and GP100 (209-217: 2M) at 2-week intervals. Participants also received twice weekly intramuscular injections of 20 mcg/kg poly-ICLC. Participants who demonstrated positive radiological response or stable disease without major adverse events were allowed to receive booster vaccines. Primary endpoints were assessments of safety and immunological responses. Clinical and radiological responses were also evaluated. To date, 8 participants (4 with glioblastoma multiforme, 3 with anaplastic astrocytoma, and 1 anaplastic oligodendroglioma) have completed the schedule of 4 vaccinations spanning 8 weeks with no major adverse events. Increased CD8+ cells reactive to HLA-A2.1-EphA2 (883-891) and HLA-A2.1-IL-13Rα2 (345-353) tetramers were detected in postvaccine peripheral blood mononuclear cells in 3 of 4 participants evaluated. These patients also demonstrated up-regulation of a chemokine receptor CXCR3 on CD8+ peripheral blood mononuclear cells following vaccines, indicating that the vaccine regimen induced type-1 CTL responses. One of these participants with recurrent glioblastoma multiforme exhibited partial radiological response, which persisted for 7 months with booster vaccines. Biopsy of tumor site after vaccination in this participant revealed intensive infiltration of CD8+ T cells and macrophages. Three of the 8 patients who received at least scheduled 4 vaccinations were progression free at 6 months. These interim data demonstrate preliminary safety and immunological activity of poly-ICLC-assisted alphaDC1-based vaccines. Most importantly, the current study demonstrates, for the first time, induction of specific reactivity against novel IL-13Rα2-derived and EphA2-derived epitopes in vaccine recipients.

Different Tumor Antigens in the Immunotherapy of Cancer: Are We Selecting the Right Target?

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Human tumor antigens (Ags) include peptides recognized by T cells in the context of class I or class II human leukocyte antigen (HLA). These Ags have been grouped according to their molecular characterization and tissue distribution. Among them, we described new mutation-derived Ags in melanoma cells and a new ubiquitary colon cancer Ag recognized by T cells of advanced but not early colon cancer patients. The group of shared/self and cancer/testis Ags have been used as vaccines in patients with different forms of cancer. While the first trials of phase I and II have been conducted with 1 or 2 such peptides, during the last few years multiple peptides have been administered simultaneously in an attempt to avoid selection of Ag-negative tumor cells by T lymphocytes elicited by vaccination. Peptide-based vaccines have been usually given emulsified in incomplete Freund's adjuvant-like adjuvant Montanide ISI 51 or pulsed onto autologous dendritic cells.

These phases I to II trials when summarized have resulted in a variable frequency (20% to 60%) of patients developing an antivaccine-specific T-cell response, while tumor regression have been reported in a minority of cases. An attempt to vaccinate patients with autologous tumor-derived gp96 heat shock proteins (possibly including mutation-derived Ags) led to tumor-specific T-cell immune response in 50% to 60% of metastatic melanoma and colon carcinoma patients with evidence of better survival in immune responders as compared to nonresponders. Recent experiments suggest that gp96 may work by a specific interaction with the CD91 receptor of plasmacytoid DCs.

A potential new target of immunotherapy is represented by cancer stem cells (CSCs). We have evaluated the antigenic profile of glioblastoma CSC, which showed impaired expression of HLA as compared with non-CSC counterparts; susceptibility to T and natural killer T cytotoxicity was also reduced. Several studies have addressed the reasons of the limited clinical outcome of vaccination. In addition to the previously defined escape mechanisms (eg, down-regulation of HLA/peptide complexes by tumor cells), it has recently been shown that new factors may prevent tumor rejection even in the presence of an ongoing tumor-specific immune reaction induced by the vaccine. These are the activation of T regulatory lymphocytes and of myeloid-derived suppressor cells. We have found myeloid-derived suppressor cells both in the blood and tumor tissue of patients with metastatic melanoma and colorectal cancer. Mechanisms underlying such suppressive activity, including the release of microvescicles, will be described. These principles have now been incorporated in designing new vaccination protocols with multipeptides in melanoma patients and early prostate cancer patients. Preliminary data of these trials will be presented.

CD8+ T Cells Activated Using a Low Affinity MHC Class I Ligand From Tyrosinase Related Protein 1 Lead to Tumor Rejection

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Vaccine strategies for cancer immunotherapy have focused on peptide ligands with high affinity for major histocompatibility complex (MHC). Largely, these vaccines have not been therapeutic. We have examined the peptide specificity of a strongly protective CD8+ T-cell response, which eliminates established B16 melanoma and find that these T cells recognize a low affinity MHC class I ligand from tyrosinase-related protein 1 (TRP1). Cytotoxic T-cell responses are induced against TRP1(222 to 229) by several vaccination schemes using toll-like receptor agonist, T regulatory cell depletion, or the immune modulator B7-DC XAb to drive immunity. TRP1-222 cytotoxic T lymphocyte (CTL) can be generated from multiple antigen sources, including antigens expressed by tumors growing in situ, tumor cell lysates, and peptide vaccines. The key finding in this study is that protection from freshly implanted or established B16 tumors is mediated primarily by TRP1-222–specific CTL and not by CTL specific for more traditional melanoma antigens such as TRP2 or gp100. This finding challenges the assumption that the optimal peptide antigens for cancer vaccines are high affinity MHC ligands. We propose that when administered appropriately, native low affinity MHC ligands are optimal inducers of immunotherapeutic CTL (Fig. 1).

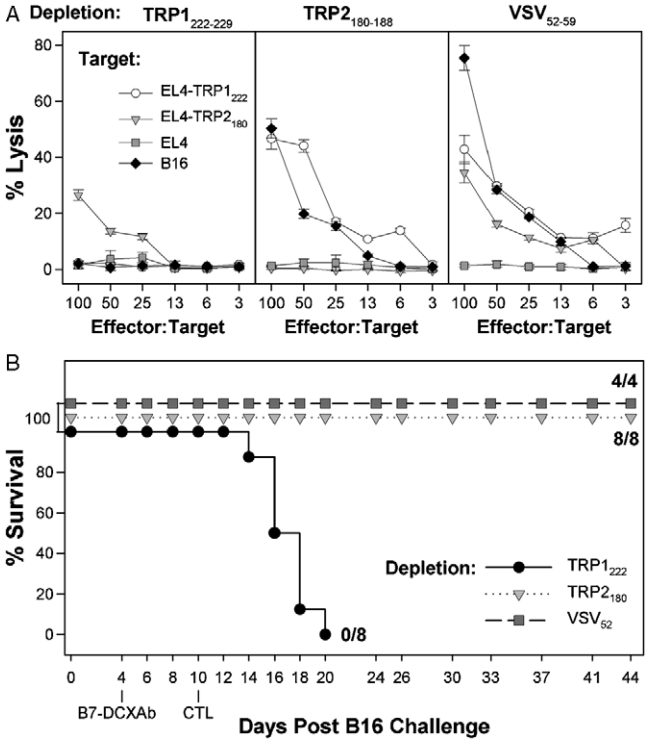

FIGURE 1.

TRP1-222–specific CTL are sufficient for clearing established B16 tumors. (A) Groups of animals were depleted with TRP1(222 to 229), TRP2(180 to 188), or VSV(52 to 59) peptides, 1 day before challenge with B16. On day 4, depleted groups were treated with B7-DC XAb and CTL activity was assessed 6 days later for specificity using B16 (diamond) and TRP1-222 (circle), TRP2-180 (triangle) pulsed or unpulsed EL4 (square). (B) Tumor challenged animals were monitored for survival ($P < 0.001$ TRP1-222 vs. VSV-52 or TRP2-180 depletion).

Allogeneic Retrovirally Transduced, IL-2 and IFN-gamma Secreting Tumor Cell Vaccine in Patients With Hormone-Refractory Prostate Cancer (HRPC)-A Phase I/II Trial

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Background: Prostate cancer (CaP) is the third leading cause of cancer death in males (Jemal, et al. *Cancer Statistics*. 2008). While treatment of organ-defined CaP is curative, treatment options for metastatic or hormone-refractory disease are limited and new therapeutic strategies are required. A clinical phase I trial was established to determine the safety and efficacy of an allogeneic CaP cell line, expressing recombinant human interleukin-2 and interferon-γ.

Methods: In a dose-escalating study, HLA-A*0201-matched hormone refractory prostate cancer patients received 4 vaccinations of irradiated allogeneic LNCaP interleukin-2/interferon-γ cells at day 1, 15, 29, and 92 and subsequently every 91 days unless tumor progression was evident. Clinical monitoring was done by serial prostate-specific antigen (PSA) measurements to determine the PSA doubling time. Secondary end point was a longitudinal evaluation of immunologic responses.
Results: The vaccine was well tolerated with no dose-limiting toxicities or autoimmunity. Only 1 patient in the higher dose level group developed a transient grade 3 injection site reaction after the eighth vaccination. Most of the patients showed lymphocytic infiltrations in delayed-type hypersensitivity challenge sites and a characteristic cytokine/chemokine profile. Vaccine-induced immune responses against a broad array of CaP associated antigens were detectable in all patients. T cells with a restricted T-cell receptor repertoire at the skin biopsy site could be observed in 1 patient with a 89% decline of the serum PSA and a long stabilization (693 d). Artificial neural network analysis was used in addition to conventional statistical methods for the analysis of the immune monitoring data and the results indicate that special immune reactivity patterns can be used to predict the response to tumor vaccination therapy.
Conclusions: This vaccine strategy showed a significant prolongation of PSA doubling time and was found to be safe and feasible. The encouraging data provides the scientific rationale for the further clinical development of the vaccine.

A Clinical Phase I Trial With an Allogeneic Gene-modified Tumor Cell Vaccine (RCC-26/CD80/IL-2) in Patients With Metastatic Renal Cell Carcinoma

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Background: Immunotherapy can induce remissions in a limited subset of patients with metastatic renal cell carcinoma (mRCC). An allogeneic gene-modified tumor cell vaccine (RCC-26/CD80/IL-2) showed enhanced immunogenic potential in several preclinical studies. Alloresponses can support development of tumor-antigen–specific T cells by providing a milieu rich in cytokines and T-cell costimulatory ligands.
Methods: For treatment of patients with mRCC, a clinical phase I trial using the tumor vaccine RCC-26/CD80/IL-2 was conducted. Patients of HLA-A*0201 allotype with histopathologically proven RCC with at least 1 evaluable metastatic lesion were included in the study. The vaccine was given up to 10 times by subcutaneous/intradermal injection during a 22-week period at increasing doses of 2.5, 10, and 40×10^6 cells. Primary end points were feasibility and safety. Clinical examination, routine blood analysis, and restaging with computed tomography and bone scan were performed according to the therapy schedule. Blood samples were collected at different time points throughout the study for the immune monitoring.
Results: Thirteen evaluable patients were enrolled in the study, 2 of whom are still alive. The median time to progression was 5.3 months; the median survival time was 17.3 months. No severe adverse events or evidence for induction of clinically relevant autoimmunity were observed. Local skin reactions increased with numbers and doses of vaccinations in 11 of 13 patients, especially in those patients with prolonged survival times. In these patients even a higher frequency of antigen-specific T-cell responses and a distinct cytokine/chemokine pattern were detectable.
Conclusions: The application of RCC26/CD80/IL-2 vaccine cells is feasible and safe in mRCC patients. Stable disease lasting up to 24 months and detectable antigen-specific T-cell responses suggest immunological activity of the RCC26 vaccine in vivo. The immunogenic potential of this vaccine and low toxicity indicate that this form of generic immunotherapy could be used in combinatorial therapies.

Telomerase-transfected Lymphocytes Stimulate Antigen-specific CD8 T Cell Responses in Patients With Stage III Melanoma

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Telomerase reverse transcriptase (hTERT) has been used by several groups as a target tumor antigen in clinical trials of cancer immunotherapy with encouraging immunologic results. We initiated an adjuvant phase II clinical trial in which a plasmid encoding for 2 HLA-A2–restricted hTERT peptide epitopes was used to spontaneously transfect peripheral blood monocytes (PBMCs) from melanoma patients ex vivo. Patients with melanoma stage III B and C had tumors resected 2 months prior to treatment with our candidate therapeutic vaccine. Patients were infused with freshly transfected autologous PBMCs then infused with 2 boosters of thawed PBMCs at 1-month intervals. In an effort to enhance the immune response, patients were treated with low-dose cyclophosphamide prior to infusion of each booster dose. PBMCs from 12 patients, collected at several time points posttreatment, were assayed directly ex vivo without in vitro sensitization. The treatment correlated with expansion of circulating CD8+ T cells specific for both p540 and pY572 hTERT peptides. These cells express markers of functional CD8+ cells: interferon-γ and perforin. These specific immune responses were maintained for longer than 3 months posttreatment. It is hoped that these promising immunological findings in our ongoing clinical trial will be supported by positive clinical results.

Contribution of the MVA-BN® Vector to the Anti-tumor Activity of MVA-BN®-HER2, a Vaccine Candidate in Development for the Treatment of Breast Cancer

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MVA-BN®-HER2 is an MVA-BN®-derived, replication-deficient vector designed for the treatment of breast cancer that is currently being evaluated in phase I clinical trials. We have previously shown that MVA-BN® induces innate immunity, which contributes to MVA-BN®-HER2 antitumor activity in a mouse model of breast cancer. Here, we present evidence that MVA-BN® may further contribute to MVA-BN®-HER2 antitumor activity by affecting the tumor immune environment and by acting as a T_H1 adjuvant for the anti-HER-2 immune response.
The immunomodulatory properties of MVA-BN® were evaluated by monitoring the recruitment of specific immune cells in organs and tumors of mice treated with MVA-BN®-HER2. Immunophenotyping of tumor infiltrating lymphocytes notably identified a large population of CD8+ CD11c+ NKG2D+ T cells in mice treated with MVA-BN®-HER2, which most likely represent a population of highly activated effector cells. Moreover, MVA-BN®-HER2 treatment induced changes of frequency and number of various T-cell subsets and immunosuppressive cell types in the tumor environment that may affect antigen-specific antitumor responses. The adjuvant activity of MVA-BN® was evaluated by characterizing the type of anti-HER-2 immune responses elicited in mice following challenge with HER-2+ tumor cells or administration of either MVA-BN®-HER2 or a recombinant extracellular domain of HER-2 in Freund's adjuvant. We found that treatment with MVA-BN®-HER2 led to anti-HER-2 antibody and T-cell responses that were more biased toward T_H1 immunity. In addition, tumor induced anti-HER-2 immune responses that were ineffective at controlling tumor growth were switched by MVA-BN®-HER2 vaccination to T_H1-biased anti-HER2 immune responses that provided effective protection. Overall our findings confirm that MVA-BN® is an efficacious delivery vector that also contributes to antitumor activity by shaping the tumor immune environment and antigen-specific responses toward effective responses for the control of tumor growth.

Establishment of Melanoma Cell Lines for Autologous Vaccine Therapy: Comprehensive Analysis of Seven Year Feasibility

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Autologous melanoma cells grown in vitro have been of considerable value for active-specific immunotherapy and subsequent monitoring of tumor immune responses as they provide all available antigens of a particular patient. In 7 years, 164 tumor tissues were processed and the resulting cell suspensions were cultured in RPMI-1640 medium containing 7.5% FBS and 7.5% iron-supplemented calf serum either fresh or after cryopreservation. Pure stable melanoma cell lines were established from 110 of 164 (67% success rate) specimens. All cell lines were subcultured efficiently after cryopreservation and expanded to $> 15 \times 10^7$ cells for making tumor cell vaccine. Fifty-four of 164 were called "no grow" after considerable culture attempts resulting in either only fibroblasts or purified tumor cells that did not grow. Of successful lines, 4 were established from pure tumor tissues and 106 were from tissues that contained tumor cells and fibroblasts. One of 110 successful cell lines was derived from primary tumor and the rest were from either metastatic (87%) or recurrent (13%) tumors. Successful melanoma lines were feasible in 82 of 122 processed specimens cultured fresh and in 28 of 42 after cryopreservation. Likelihood of establishing melanoma lines were between 55% and 80% with different tissue sites, skin (55%), lymph node (69%), liver (64%), lung (71%), chest wall (75%), bone (80%), and other tissue sites (73%). Overall, mean time taken to establish successful melanoma line was 3.1 ± 2.5 months. The pattern of expression of S-100, HMB45, MART-1/Melan-A, tyrosinase, TRP-1/TRP-2, and MAGE-1 in 105 lines, as determined by immunocytochemistry, revealed that all tested antigens expressed in only 9 of 105 lines, in the range of $< 1\%$ to $> 75\%$ positive cells. As a major antigen, tyrosinase was expressed in 83% of the lines. MART-1/Melan-A was expressed in 81% lines with $> 25\%$ positive cells in 64 and few to 25% positive cells in 21 lines. Expression of HMB45 was seen in 75% lines with $> 25\%$ positive cells in 57 and few to 25% positive cells in 22 lines. Mel-5 was expressed in 66% lines with $> 25\%$ positive cells in 37 and few to 25% positive cells in 32 lines. MAGE-1 expressed in 48% lines with $> 25\%$ positive cells in 22 whereas few to 25% positive cells in 28 lines. S-100 expressed in 29% lines with $> 25\%$ of positive cells in 17 and few to 25% positive cells in 13 lines. In an exclusive pattern, MAGE-1 was expressed by 3, tyrosinase was by 2, and Mel-5 was by 1 cell line. In summary, our findings suggest that melanoma cell line containing heterogeneous tumor cell clones with diverse tumor-associated antigens of a given tumor tissue can be reliably established from high number of tumor tissue samples in order to make autologous vaccine.

Interleukin-15 and Its Receptor Enhance Antitumor Activity Following a Genetically Modified Dendritic Cell Vaccine

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Background: Interleukin-15 (IL-15) has been shown to induce T and natural killer cell proliferation and differentiation, enhance cytolytic effector cells including antigen-experienced CD44hi CD8+ T memory cells, and enhance B-cell stimulation. Unlike IL-2, IL-15 does this without inducing T regulatory cells or stimulating activation-induced cell death thus making IL-15 an ideal adjuvant candidate for cancer vaccines. IL-15 functions through interaction with its receptor (IL-15Rα), presenting IL-15 in trans to immune effectors cells. The efficacy of exogenously administered IL-15 may be limited by the availability of IL-15Rα therefore a genetic vaccine expressing both the cytokine and its receptor may be advantageous. BALB-neuT transgenic mice develop breast cancers as a consequence of mammary gland-specific expression of an activated *neu* oncogene. We examined the antitumor effect of adenoviral-mediated gene transfer of the combination of IL-15 and IL-15Rα to augment a dendritic cell (DC) vaccine directed against the *neu* oncoprotein in these mice.

Methods: Bone marrow-derived DCs were generated from BALB/c mice and transduced with recombinant adenoviruses expressing a nonsignaling truncated *neu* antigen, murine IL-15 and its receptor, IL-15Rα. Transgenic BALB-neuT mice at 10 to 12 weeks of age were subcutaneously vaccinated with 4 weekly injections of 1×10^6 genetically modified DCs and followed for tumor development and immune response.

Results: Mice vaccinated with IL-15, IL-15Rα, and the *neu* antigen, were protected from the onset of mammary carcinomas with 70% of animals tumor free at 25 weeks compared to 10% of animals treated with DC expressing the *neu* antigen alone, and none of the unvaccinated control mice. These mice also exhibited greater tumor protection than mice vaccinated with *neu* and either IL-15 (30% tumor free) or IL-15Rα (40% tumor free) alone. The combination of IL-15 and IL-15Rα lead to significantly greater antibody responses to the *neu* antigen compared to mice treated with DCs expressing *neu* alone, or *neu* combined with IL-15 or IL-15Rα alone. Serum from vaccinated mice exhibited antibody-dependant cellular cytotoxicity, complement-dependent cytotoxicity against *neu*-expressing target cells and induced down-regulation of *neu* signaling in vitro.

Conclusions: Co-expression of IL-15 in combination with its receptor augments antitumor vaccination with genetically modified DCs expressing the *neu* antigen highlighting the potential for the use of IL-15 and IL-15Rα gene transfer as an adjuvant for anticancer vaccination.

In Vivo Application of Biodegradable Artificial Antigen-presenting Cells

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T-cell–mediated immunotherapy requires expansion and activation methods that are both highly effective and optimally efficient. By including all necessary signals for T-cell activation in a single artificial antigen-presenting cell (aAPC) construct that rapidly expands T cells, we have met both of these criteria. Our polymeric aAPC are biodegradable and composed of FDA-approved materials, making them safe and effective when used in vivo for active immunotherapy or in vitro to expand T cells for adoptive immunotherapy. The biodegradable nature of these cell-sized particles allows for the encapsulation and controlled release of soluble immune mediators, such as the cytokine interleukin-2, from assembled aAPC. We have shown previously that releasing interleukin-2 in this paracrine manner from T-cell–targeted aAPC results in qualitative and quantitative differences in CD8 T-cell activation and expansion compared to addition of soluble cytokine to cultures. We now present evidence explaining the underlying mechanism of this paracrine effect from polymeric aAPCs in vitro as well as a demonstration of the efficacy of this system in vivo using the B16 mouse melanoma model.

Neutrophil-like Myeloid Derived Suppressor Cells are Increased in Heavy Tumor Burden and Upregulate MHC Class II Expression in Response to Toll-like Receptor Stimulation

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Myeloid-derived suppressor cells (MDSCs) are heterogeneous, immature cells that have been shown to inhibit T and natural killer cell activity in tumor-bearing hosts. MDSCs do not express major histocompatibility complex (MHC) class II and are not known to participate in antigen presentation. Toll-like receptor (TLR) agonists are being investigated in cancer immunotherapy regimens to activate antigen-presenting cells but their effect on MDSCs is unclear.

We sought to characterize the MDSC population in 2 clinically relevant mouse models of heavy solid tumor burden and to assess the effect of TLR stimulation on MDSCs.

CT26 mouse colon adenocarcinoma cells were injected into the peritoneum or spleen of BALB/c mice to establish carcinomatosis or liver metastases, respectively. When mice became moribund, they were euthanized and peripheral blood was collected by cardiac perfusion, and mesenteric and portal vein lymph nodes were harvested. Mononuclear cells were stained with fluorochrome-conjugated antibodies against

various cell surface markers and analyzed by flow cytometry. In a separate experiment, Ly6G-positive cells from the peripheral blood of CT26 carcinomatosis mice were isolated using magnetic beads and plated in media supplemented with granulocyte macrophage-colony stimulating factor. Plated cells were then stimulated for 48 hours with the TLR agonists, lipopolysaccharide, or CpG. Aliquots of cells were taken before and after stimulation and stained for the same panel of surface markers.

MDSCs (Gr-1+, CD11b+) were expanded 40-fold in the peripheral blood of mice with carcinomatosis and 10-fold in mice with liver metastasis. In contrast, no MDSCs were seen in the intra-abdominal lymph nodes in either model. MDSCs were negative for expression of MHC class II as well as markers for dendritic cells (CD11c), monocyte/macrophages (F4/80), and B cells (CD19). The vast majority of the MDSC population strongly expressed the neutrophil marker, Ly6G. When stimulated with TLR agonists in vitro, a subpopulation of Ly6G-positive MDSCs differentiated into macrophage-appearing cells with high expression of MHC class II. In a dose-dependent manner, LPS expanded this population 2-fold, while CpG expanded this population 5-fold.

Our results suggest that under the appropriate conditions, MDSCs can differentiate into cells that may have the potential to present antigen and initiate an antitumor response. Alternatively, if antigen is presented inappropriately, these differentiated MDSCs may further promote tumor tolerance. Given the dramatic expansion of MDSCs in heavy tumor burden, the potential to convert an immunosuppressive population into cells with antitumor activity deserves further exploration.

Development of Multiple Listeria Monocytogenes Based Constructs for the Therapeutic Treatment of Prostate Cancer

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Prostate cancer remains a major health problem, especially in United States, resulting in over 30,000 deaths in men annually. Cancer vaccines are a new therapeutic concept and are being rapidly evolved to battle various types of cancer, specifically those presenting specific tumor antigens. Prostate-specific antigen (PSA) is one of the most favorable target antigens and has been used in various immunotherapeutic approaches against prostate cancer. PSA is mostly expressed by prostate epithelial cells and is overexpressed by prostate carcinomas. Previously, we have shown that Listeria monocytogenes (Lm) is an effective vector for the regression of tumors in mice that were implanted with a genetically modified mouse prostate adenocarcinoma cell line, which expressed human PSA, TPSA23. We constructed a series of Lm strains that display different levels of attenuation and were expressing PSA either episomally or chromosomally. We examined the antitumor therapeutic efficacy of these constructs using our previously described mouse model, TPSA23 and our results indicate that Lm-based constructs expressing PSA can efficiently cause the regression of TPSA23 tumors in mice. In addition, all the constructs elicited strong PSA-specific immune responses in mice when examined by tetramer staining and intracellular cytokine staining. Thus, these results prove that Lm-PSA has viable potential to be taken forward for further testing in human clinical trials.

The 1170 A-P Small Nuclear Polymorphism (SNP) in the HER-2/NEU Protein (HER2) as a Minor Histocompatibility Antigen (MHAG)

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Allogeneic hematopoietic cell transplantation represents a potentially curative treatment modality for several hematologic malignancies. T-cell responses to minor histocompatibility antigens (mHags) are at least in part responsible for graft-versus-leukemia as well as graft-versus-host effect. Here, we report the identification of a novel mHag encoded by the 1170 P→A single nuclear polymorphisms in the Her-2/neu (HER2) tumor-associated antigen. Hence, an HLA-A2–restricted cytotoxic T lymphocyte clone specific for the P-peptide kills homozygous P/P as well as heterozygous A/P cancer cells; homozygous A/A cancer cells were not killed by the P-peptide–specific cytotoxic T lymphocyte unless loaded with exogenous P-peptide. Importantly, substantiating the in vivo relevance, ex vivo sorted acute myeloid leukemia cells were lysed exclusively if the P allele were expressed. Since a substantial fraction of cancer cells express high levels of HER2 in contrast to most normal cells, the described mHag could be used as a means to direct the hematopoietic cell transplantation-induced immune response in a graft-versus-leukemia direction.

T Cell Receptor-dependent and Independent Pathways Control PD-1 Expression on CD8+ T Cells Generated Upon Intra Lymph Node Immunization

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Programmed death-1 (PD-1) has been shown to be a marker for T-cell activation; however, persistently elevated PD-1 expression is associated with T-cell exhaustion, potentially a barrier to achieve optimal immunity against viruses and cancer antigens. Using a direct lymph node-targeted vaccination procedure that allows uncoupling of signal 1 (T-cell receptor-mediated) versus signal 2 (non–T-cell receptor-mediated), we evaluated the impact of antigen-dependent and antigen-independent signals on epitope-specific CD8+ T-cell–associated PD-1 expression. The level of antigen exposure and costimulation mediated by CpG oligodeoxynucleotide (ODN) TLR9 agonist respectively, had dramatic yet opposite effects on overall PD-1 acquisition by specific CD8+ T cells. For example, high-dose antigen exposure with minimal immune costimulation yielded CD8+ T cells with significantly elevated PD-1 expression. This was associated with impairment of interferon-γ secretion and proliferation in vitro, reversible upon antibody-mediated PD-1 blockade. By comparison, low antigen exposure in context of increased immune costimulatory signals—for example low-dose peptide+CpG ODN adjuvant or DNA plasmid vaccination, respectively—yielded CD8+ T cells with low PD-1 expression, greater in vitro

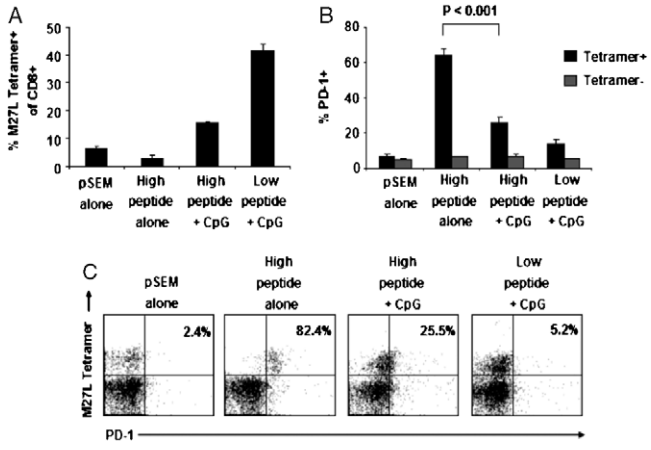

FIGURE 1. PD-1 expression profile of peripheral blood epitope-specific CD8+ T cells elicited by DNA and peptide vaccination in HLA-A*0201 transgenic HHD-1 mice. A, M27L-specific immune response magnitude. B, PD-1 expression on M27L tetramer+ and tetramer– CD8+ T cells. C, Representative dot plots for M27L-specific CD8+ T cells. Upper right quadrants display percent of tetramer+ cells that are PD-1+. All data are representative of ≥2 independent studies. N=10/group; pSEM, DNA plasmid encoding human Melan A; M27L, human MelanA26-35(27L); Error bars, SEM.

proliferative capacity, and increased interferon-γ secretion upon stimulation with cognate antigen. These findings shed light on molecular mechanisms involved with the homeostasis of CD8+ T cells and elucidate key features of DNA and similar vaccines that are currently investigated in several clinical trials (Fig. 1).

MelCancerVac®: Autologous Dendritic Cells Pulsed With an Allogeneic Tumor Lysate for the Treatment of Colorectal Cancer and Non Small Cell Lung Cancer

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The ability of dendritic cells (DC) to induce a strong immune response is the basis for vaccine therapies at DanDrit Biotech A/S, Denmark. Our expertise in producing DC from patient blood monocytes is combined with conventional production methods to make new and advanced vaccines for cancer patients especially for MAGE-A–positive tumors in colorectal cancer (CRC) and nonsmall cell lung Cancer (NSCLC). Our best clinical results have been obtained in trials where our second-generation DC production method was used. This method generates highly matured DC (CD83 high, CD86 high, major histocompatibility complex class I/II high) in combination with high expression of CCR7. In patients, phenotypic DC variations have been detected with both our first and our second-generation method in which some DCs still exhibit a monocytic profile (high expression of CD14) even after addition of the maturation cocktail at day 6 of culture thus reflecting that monocytes from some patients are not able to differentiate in vitro. We are currently testing which markers could correlate with the inability to induce mature DC. When our DC second-generation method was used and MAGE-A expression in CRC tumor biopsies was used as patient inclusion criteria, the clinical results showed response rates of 35%. In our NSCLC trial in which MAGE-A expression is not used as an inclusion criterion, we are conducting a retrospective study on MAGE-A antigen expression in order to understand the relevance of this antigen group for the MelCancerVac® efficacy.

In order to establish a potency assay for vaccine-lysate pulsed DC, we have generated a number of major histocompatibility complex class I and II restricted® MelCancerVac®-specific T-cell clones from a patient in our NSCLC trial. Some of these T-cell clones react with tumor cell lines expressing vaccine-relevant rejection epitopes. The T-cell clones will be used to monitor the expression of tumor antigens in MelCancerVac®—a potency assay system, which is required by the regulatory authorities.

Taken together, we are encouraged by the clinical results and aim toward a larger multicenter study in the CRC group of patients.

Please take a look at *www.dandrit.com* for a review of our clinical trials and data.

INFECTIOUS AGENT VECTORS

A Phase I Study to Evaluate Systemic Wild-type Reovirus (REOLYSIN)® in Combination With Docetaxel in Patients With Advanced Malignancies

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Wild-type reovirus (REOLYSIN)® serotype 3 Dearing has significant antitumor activity in vitro, in vivo, and after systemic delivery in humans in a recently completed trial. Synergistic tumor kill has been observed combining reovirus with radiotherapy and chemotherapy, and in particular with taxanes in a range of cancer models, justifying clinical evaluation of the combination.

Methods: This dose escalating, phase I study combined 3 weekly 75 mg/m^2 docetaxel (TAX) intravenously with 3 weekly (day 1 to 5 of first week inclusive) intravenous reovirus. Reovirus was increased in 3 cohorts of 4 patients with doses (all tissue culture infectious dose, TCID50) from 3×10^9, to 1×10^{10} and 3×10^{10}. Patients with ECOG performance status ≤ 2, and evaluable metastatic solid tumors refractory to standard therapy, for whom TAX was an appropriate chemotherapy, were eligible. The 1o end points were to determine maximum tolerated dose, dose limiting toxicity of combination; 2o end points to evaluate safety, viral replication, viral shedding, immune response.

Results: Twelve patients were treated (10 males, 2 females): mean age 56 and mean performance status of 2. Tumors treated included prostate (3), gastric (1), esophageal (1), mesothelioma (1) nonsmall cell lung (1), breast (1), unknown 1o (1), melanoma (1), hepatoma (1), and pancreas (1). Three patients completed 6 cycles, 1 patient is still on treatment at cycle 7 and 4 others ongoing between cycle 1 and cycle 4. Maximum tolerated dose was not reached. We observed 1 complete resolution of the target lesion in breast (with stable disease of nontarget lesions); 1 partial response in gastric; and 2 stable diseases in lung and melanoma. Myelosuppression consistent with the effects of TAX was observed (3 grade IV toxicities with neutropaenic sepsis): 1 patient in cohort 3 had a recrudescence of a perianal abscess with grade IV neutropenia. One patient had a grade 3 rise in aspartate transaminase possibly associated with combination, which had resolved. Three more patients are now being treated at the highest dose level. We will look for viral shedding in blood, sputum, urine and stool; induction of antireoviral antibodies and evidence of viral replication from pretreatment and posttreatment tumor biopsies.

Conclusions: Reovirus can be safely combined with TAX chemotherapy. The significant toxicities observed were consistent with those expected with TAX alone. There was objective radiological evidence of anticancer activity, and phase II studies with this combination are justified.

Synergistic Anti-Tumor Activity of Oncolytic Reovirus and Docetaxel in a PC-3 Prostate Cancer Mouse Model

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Background: The oncolytic potential of reovirus type 3 Dearing (T3D) has been demonstrated in numerous in vitro systems and in vivo murine models, and is currently undergoing clinical evaluation. Combination of reovirus with radiotherapy and chemotherapy may enhance tumor kill. In this study, we examine the in vitro and in vivo oncolytic activity of T3D against the human prostate cancer (PC) cell line PC-3 in combination with docetaxel. Docetaxel is the standard of care for metastatic PC, and acts by blocking microtubule polymerization in PC cells. Although infection by type 1 Lang strain of reovirus is associated with microtubule stabilization, the key μ2 proteins from T3D infection do not co-localize with microtubules suggesting the possibility of synergistic antitumor effects between T3D infection and antitubular chemotherapy agents.

Methods: Using a constant ratio combination design and the combination index method based on the Chou and Talalay median-effect principle, the effect of reovirus combined with docetaxel, paclitaxel (another chemotherapeutic of the taxane family) or vincristine (a non-taxane microtubule assembly inhibitor) on PC-3 was assessed. Apoptosis induction following exposure to reovirus and/or docetaxel was measured using flow cytometry. In the in vivo setting, PC-3 tumors were seeded subcutaneously in nude mice and treated with intratumoral reovirus T3D 5×10^8 TCID50 on day 1 and 4 and intraperitoneal docetaxel 5 mg/kg on day 1 and 4 either alone or in combination, or with control treatment (intratumoral PBS, intraperitoneal PBS).

Results: A synergistic interaction (combination index value of less than 1) was observed between reovirus and each of the chemotherapeutic agents, except for docetaxel at very high concentrations.

Flow cytometric analysis showed a marked increase in apoptotic cells following combined exposure, compared to single agent exposure. Combined T3D/docetaxel treatment resulted in markedly reduced tumor

growth compared to single agent treatments. Mean relative tumor volumes ± SEM day 11–control 4.69 ± 1.38, reovirus T3D alone 1.72 ± 0.91, docetaxel alone 4.45 ± 1.27, reovirus T3D plus docetaxel 0.58 ± 0.37.
Conclusions: Taken together, these results indicate synergistic antitumor effects between docetaxel and reovirus therapy and supports the ongoing clinical trials using these agents.

Chemotherapeutic	ED50	ED75	ED90
Docetaxel	0.41 ± 0.07	0.75 ± 0.04	1.58 ± 0.08
Paclitaxel	0.57 ± 0.01	0.40 ± 0.03	0.31 ± 0.04
Vincristine	0.46 ± 0.06	0.34 ± 0.05	0.44 ± 0.11

Data is presented as CIV ± SEM at the effective dose indicated.

Phase I Trial of Oncolytic Reovirus (Reolysin) in Combination With Carboplatin/Paclitaxel in Patients With Advanced Solid Cancers

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Following our recent study of intravenous Reolysin (reovirus Type 3 Dearing, Oncolytics Biotech. Inc, Calgary, Canada) in patients with advanced malignancies (Vidal, et al. *Clin Cancer Res*. In press), we have conducted a phase I dose-escalation study of Reolysin in combination with carboplatin (AUC 5) and paclitaxel (175 mg/m^2 over 3 h). Reolysin was administered to cohorts of 3 patients in escalating doses (3×10^9, 1×10^{10} and 3×10^{10} TCID50 on days 1 to 5 of a 3-week schedule). After completion of the dose-escalation phase, recruitment of a safety cohort has commenced. In the dose escalation phase of the study, there were no dose-limiting toxicities. Eleven patients (6 males, median age 56 years, median performance stage = 0) with head and neck cancer (n = 6), melanoma (n = 3), and peritoneal/endometrial cancer (n = 2) have received 46 cycles of treatment. Grade 3 toxicities included neutropenia (38%), lymphopenia (63%), thrombocytopenia (25%), and hypotension (13%). Viral shedding (as assayed by reverse transcription polymerase chain reaction) has been documented in 2 patients. Neutralizing antireoviral antibody responses have been measured using a previously reported technique (White, et al. *Gene Ther*. 2008;15:911–920) and response rates reported in 10 evaluable patients were partial response (3 patients), stable disease (5 patients), and progressive disease (2 patients). On the basis of promising response data in the patients with head and neck cancer, a phase II study has been opened in this indication using the 3×10^{10} TCID50 dose on 5 consecutive days.

INNATE IMMUNITY TO TUMORS

Innate Immunity can Contribute to the Shaping of Tumor Immunogenicity in the Absence of Adaptive Immunity

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Although components of innate and adaptive immunity have been shown to work together to protect the host against cancer development and sculpt tumor immunogenicity (ie, promote cancer immunoediting), it remains unclear whether innate immunity is capable of manifesting tumor-editing functions on its own. In particular, whereas natural killer (NK) cells can promote tumor surveillance of methylcholanthrene (MCA)-induced sarcomas in the context of an intact immune system, it has not been shown whether NK cells can impact on cancer immunoediting without subsequent contributions from T cells. To address this question, we compared the immunogenicities of sarcoma cells derived from MCA-treated wild type (WT) immunocompetent mice, RAG2 − / − mice lacking adaptive immunity, or RAG2 − / − × IL-2Rgc − / − mice which lack NK cells and adaptive immunity. To measure immunogenicity, MCA-sarcoma cell lines were transplanted into naive, syngeneic WT or RAG2 − / − mice to assess their ability to grow in the presence of the full immune system or solely the innate immune system. Consistent with our previous reports, all sarcoma cell lines from MCA-treated WT mice were poorly immunogenic and grew progressively when transplanted into syngeneic WT recipients. In addition, 40% of the sarcoma cell lines from MCA-treated RAG2 − / − mice displayed high immunogenicity and were rejected. Interestingly, MCA-sarcoma cells from RAG2 − / − × IL-2Rgc − / − mice were highly likely to be immunogenic since 60% were rejected when transplanted into WT mice. When these cell lines were transplanted into RAG2 − / − mice, all cell lines were able to grow. However, the RAG2 − / − × IL-2Rgc − / − MCA-sarcomas displayed delayed growth compared to MCA sarcomas from RAG2 − / − and WT mice. Furthermore, RAG2 − / − × IL-2Rgc − / − tumors that were transplanted into RAG2 − / − mice became heavily infiltrated with innate immune cells that expressed high levels of MHC class II. This infiltration required IL-2Rgc function, suggesting that NK cells are important in the recruitment of class II+ cells into highly immunogenic tumors. Finally, the infiltration of innate cells into highly immunogenic tumors resulted in tumor editing, since highly immunogenic tumor cells that were passaged through RAG2 − / − but not RAG2 − / − × IL-2Rgc − / − mice became poorly immunogenic. Our results conclusively show that innate immunity can manifest IL-2Rgc–dependent tumor editing function in the absence of adaptive immunity.

Breast Tumor Growth and Macrophage: The Effect of Diazepam

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Background: Among the receptors described for benzodiazepines, peripheral type binding sites (PBR) has also been identified in immune organs and cells, such as macrophages and lymphocytes. PBR activation was reported to decrease innate immunity and host defense. The present experiment was designed to analyze the effects of diazepam on breast tumor growth, and on macrophage activity of breast tumor bearing animal model.
Methods: Diazepam 3.5 mg/kg/d for 2 weeks was administered daily to selected animal model and the findings were analyzed by comparing the 2 groups (1) those receiving diazepam 3.5 mg/kg/d and (2) those receiving diazepam 2 to 2.5 mg/kg/d.
Results: The findings showed that the breast tumor cells increased with the administration of diazepam (3.5 mg/kg/d, for 14 d) and the increase in breast tumor cells was not observed with the administration of diazepam (2 to 2.5 mg/kg/d). It shows that such effects are not observed after smaller doses of diazepam, which suggests that it is a dose-dependant effect. The other findings showed that when 3.5 mg/kg of diazepam was administered daily for 3 days, there was decrease in: (1) the number of leukocytes retrieved after injection of the breast tumor, (2) the percents of macrophage spreading, and (3) the levels of macrophage nitric oxide production. There was no effect on macrophage phagocytosis when diazepam (3.5 mg/kg/d for 3 d) was administered.
Conclusions: The study data shows the findings, which are based on a direct and/or indirect action of diazepam. Particularly, our findings might be due to a direct effect of diazepam on PBRs present on macrophages and tumor cells.

Completion of Taxane Treatment in Breast Cancer Patients and the Effect on Cellular Immunity

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Background: Breast cancer is one of the major diseases among the Nepalese women with positive family history. A follow-up study was conducted postchemotherapy with taxanes to find the immune functioning status after the use of taxanes. Immune responses in breast cancer patients were analyzed as a function of whether patients received taxane as part of their adjuvant chemotherapy.
Methods: The study comprises 150 patients with positive family history of breast cancer. After the surgery was performed, the immune levels of

207 stage II/III breast cancer patients were measured. This measurement was done prior to chemotherapy and was recorded. After completion of all the chemotherapy regimens, a year later another measurement was done. The levels of T-cell blastogenesis and natural killer (NK) cell lysis of both patients receiving taxanes (n = 50) and those not receiving taxanes (n = 100) were compared.

Results: The findings were compared after a regression analysis was done. The findings suggest that there was increased level of T-cell blastogenesis after the use of taxane as part of combination chemotherapy. The NK cell cytotoxicity after the conclusion of all chemotherapies was much higher as compared to those patients who did not receive taxane for chemotherapy. For those patients who received taxane, the average phytohemagglutinin-induced blastogenesis was 37% higher and NK cell cytotoxicity was 39% higher than the values for those who did not receive taxane.

Conclusions: The findings from the data with comparisons of taxane receiving group and non-taxane receiving group with appropriate controls in a sizable clinical sample suggest that taxanes suppress patients' immune cell functions. Problems in generalizing direct-contact laboratory models to the field of cancer treatment are highlighted.

Spontaneous CTL-mediated Rejection of GP33-positive Lewis Lung Carcinoma is Dependent on an IFNAR Competent Environment

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Recent evidence accumulated that interferon-alpha/beta (IFN-a/b) can support antitumor activity by stimulating host cells instead of exhibiting antiproliferative effects on tumor cells. We addressed the role of the IFN-a/b system in a model of a spontaneous tumor regression, that is, Lewis lung carcinoma expressing the cytotoxic T lymphocyte (CTL) epitope 33 of lymphocytic choriomeningitis virus glycoprotein as a tumor associated neo-antigen (A9GP33). In A9GP33 treated wild-type (WT) mice, small tumors developed within 5 to 8 days that usually were rejected by GP33-specific CTL around day 14. In contrast, mice devoid of a functional IFN-a/b receptor (IFNAR −/−) showed progressive A9GP33 growth. The analysis of such tumor cells in a GP33-specific in vitro CTL assay revealed that tumor cells were still GP33-positive. Furthermore, approximately 20% of A9GP33 tumors grown in IFNAR −/− mice were still rejected when reinjected into WT mice. Interestingly, T-cell priming was not impaired in IFNAR −/− mice as indicated by similar cytolytic activities in spleen cells of A9GP33-treated IFNAR −/− and WT mice in a secondary 51Cr release assay. Nevertheless, reduced in vivo killing of GP33-positive target cells was observed in A9GP33-treated IFNAR −/− mice when compared to WT mice. The analysis of conditional mice with a lymphocyte-specific IFNAR ablation indicated that direct IFNAR trigging of B and/or T cells did not play a crucial role in A9GP33 tumor rejection, whereas stimulation of dendritic cells was critical. Thus, our data indicate that tumor-induced CTL priming was overall normal in IFNAR −/− mice, whereas an IFNAR competent environment was required to promote efficient tumor lysis.

Gauging Innate Immunity in HIV Infection

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Human Immune deficiency virus (HIV) infection is characterized by the presence of T-cell responses that do not lead to viral clearance; little is known about baseline and stimulation-induced innate immune activation. We compared the transcriptional profile of peripheral blood mononuclear cells from normal non–HIV-infected volunteers and HIV-infected patients with low (< 2.6 log RNA copies/mL) or high viral load (> 4.69 copies/mL). Each cohort was stimulated with 6 μg/mL HIV-1 Pr55gag virus-like particles (VLPs) or 1 μg/mL lipopolysaccharide (LPS).

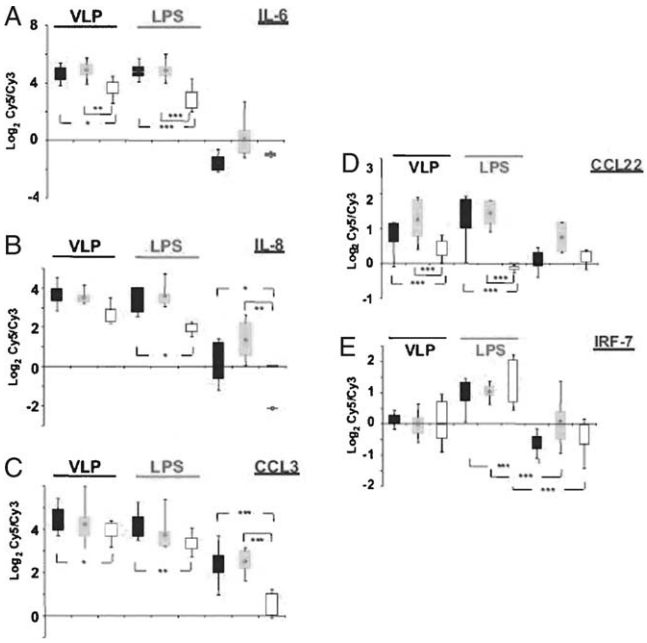

FIGURE 1. A–E, Box and whiskers charts showing relative Cy5/Cy3 values of IL-6, IL-8, CCL3, CCL22, and IRF-7. Data represent cumulative Cy5/Cy3 values for each experimental category and P values are represented as asterisks (unpaired Student *t* test; *, **, ***P value <0.05, <01, and <0.001, respectively). Black, Gray, and white boxes indicate high viral load, low viral load, and healthy normal controls. VLP and LPS treatments are indicated respectively by blue and orange bars. LPS indicates lipopolysaccharide; VLP, virus-like particle.

Results show that (1) VLPs induce in vitro transcriptional activation similar to a constitutive immune activation induced by live HIV in vivo suggesting that immune activation is independent of viral replication and transcription; (2) The constitutive activation of immune genes observed in vivo in HIV-infected patients is further enhanced by ex vivo VLP stimulation resulting in transcriptional activation superior to that observed in peripheral blood mononuclear cell obtained from normal non–HIV-infected volunteers; (3) the response of HIV-infected patients to LPS is not diminished compared to non–HIV-infected donors and follows a patient-specific pattern rather than HIV viral load and severity of disease; (4) endogenous HIV infection or exogenous VLP stimulation results in transcriptional patterns overlapping with LPS stimulation with a dramatic difference in activation of interferon-stimulated genes uniquely induced by LPS (Fig. 1). Lack of interferon-stimulated gene activation may explain the limited effectiveness of immune responses against HIV and explain their limited effectiveness in other rejection models including cancer.

TLR Engagement as a Mechanism of Prevention of Tumorigenesis

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Toll-like receptors (TLRs) are one of the pattern recognition receptors that identify conserved molecular signatures of microbial origin, called the pathogen-associated molecular patterns. Role of TLRs in allergy development and atopy has been proposed in the hygiene hypothesis, which states that "lack of early childhood exposure to infectious agents, symbiotic microorganisms, and parasites increase susceptibility to allergic diseases by modulating immune system development." Epidemiological data suggests that the cancer prevalence is higher in developed countries (like the US and Australia) when compared to underdeveloped and developing countries (like the countries in Africa

and South East Asia). This has been attributed to the lifestyle, but we hypothesize that it could be correlated to the early exposure pattern of the individual to microbial components and engagement of TLRs on the immune cells and subsequently preparing the immune system to fight infection and cancer.

To test our hypothesis, we employed a mouse model. Age-matched and sex-matched C57BL6 mice were divided into 2 groups. These mice were housed in 2 different environments, 1 an ultra-clean environment, where cages, bedding, food, and water needed for the mice were sterilized. The other was the dirty environment, which was simulated by exposing the mice to cocktail of TLR ligands (containing antigenic components of virus, bacteria, and fungi) periodically, starting from day 2 after birth. These mice were subjected to tumor challenge with an adenocarcinoma cell line (MC-38) after 12 weeks.

Results from our studies suggest that exposure of the neonatal immune system to TLR ligands, skews the immune system to T_H1 response. The analysis of the function of CD 8 T cells revealed its better cytokine producing ability (measured in terms of its ability to produce interferon-γ, tumor necrosis factor-α, and interleukin-2) and cytotoxicity (CD107 a/b, perforin, and granzyme producing ability). The natural killer cells and dendritic cells were also functionally enhanced in the mice whose immune system had been stimulated from birth. Finally, the mice that had been exposed to TLR ligands from birth were better in resisting tumor challenge with MC-38 cells in their adult life, by about 35% when compared to the unmanipulated group.

We conclude that hypersanitization and lifestyle may be a probable cause of higher rates of cancer in developed countries and the engagement of pattern-recognition receptors (TLRs) intermittently keeps the immune system alert.

Acknowledgments: Department of Microbiology, ETSU-Start-up funds; RDC Grant, ETSU. Dr Gregory Hanley and Janice Robin, DLAR, ETSU.

Enhanced T Cell-Independent Anti-tumor Effect of Cyclophosphamide Combined With Anti-CD40 MAB and CpG

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We have previously demonstrated T-cell–independent antitumor effects of a combination of anti-CD40 mAb and CpG oligonucleotides, which involved macrophages. In this study, we tested if cyclophosphamide (CY) would enhance the antitumor effect of anti-CD40 mAb + CpG. Treatment of B16 melanoma-bearing mice with CY and anti-CD40 mAb + CpG resulted in a significant reduction in tumor growth in immunocompetent mice compared to either CY alone or anti-CD40 mAb with CpG. This enhanced antitumor effect was maintained in SCID mice, with regard to both tumor growth and overall survival. Natural killer cells were not required for this antitumor effect, as it was also observed in SCID/beige mice. Moreover, while CY treatment suppressed natural killer cell activity, it did not negatively affect the antitumor activity of macrophages in vitro. These results suggest that the therapeutic strategies of activating macrophages may have potential for use in cancer patients during chemotherapy.

Role of NKG2D in Tumor Surveillance

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Natural killer (NK) cell receptors regulate the capacity of NK cells and in some cases T cells to attack tumor cells and infected cells. Diseased cells in the body become susceptible to NK cells by down-regulating inhibitory ligands such as major histocompatibility complex class I molecules, and/or up-regulating stimulatory ligands, such as the Raet1 family proteins recognized by the NKG2D receptor. This presentation will discuss the role of NKG2D in tumor surveillance in vivo and the molecular mechanisms and signaling pathways responsible for induction of NKG2D ligands in cancer cells and their relationship to major pathways regulating tumorigenesis. Supported by grants from NCI, NIAID, and Prostate Cancer Foundation.

Predicting the Immunologic Constant of Rejection

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Based on hypothesis-generating clinical studies, we postulated that immune-mediated cancer rejection is part of a broader phenomenon shared by autoimmunity, allograft rejection, and clearance of pathogens that we called "immunologic constant of rejection" (ICR).[1] ICR includes the combined expression of interferon-stimulated genes and immune effector functions.

Here, we tested the predictive value of genes associated with the ICR using GLV-1h68, an attenuated recombinant vaccinia virus (VACV)

TABLE 1. Immune Genes Up-regulated in Regressing GI-101A Tumors (*F* test *P*2 value <0.001)

Symbol	Name	HT-29 Control	HT-29 GLV-1h68	GI-101A Control	GI-101A GLV-1h68
Interleukins and Receptors					
Il18bp	Interleukin-18 binding protein	1.31	2.18	1.00	**13.28**
Il18	Interleukin-18	1.12	1.40	1.00	**10.89**
Il15	Interleukin-15	1.02	1.51	1.00	**5.20**
Chemokines					
Cxcl11	I-TAC	0.92	1.75	1.00	**13.57**
Cxcl9	Mig	1.01	1.07	1.00	**11.74**
Cxcl12	SDF-1/PBSF	0.41	0.58	1.00	**5.23**
Ccl9	MRP-2/CCF18/MIP-1 g	1.56	3.14	1.00	**12.03**
Ccl5	RANTES	1.05	2.63	1.00	**11.57**
ISGs					
Igtp	Interferon-γ induced GTPase	1.15	3.31	1.00	**48.21**
Ifi27	Interferon-α inducible protein 27	0.76	0.91	1.00	**12.84**
Ifi47	Interferon-γ inducible protein 47	0.66	0.94	1.00	**11.09**
Iigp2	Interferon inducible GTPase 2	0.64	1.41	1.00	**10.05**
Mx1	Myxovirus (influenza virus) resistance 1	0.62	1.46	1.00	**9.46**
Aif1	Allograft inflammatory factor 1	0.90	1.33	1.00	**8.46**
Irf1	Interferon regulatory factor 1	0.59	0.98	1.00	**7.28**

ISG indicates interferon-stimulated gene.

that selectively colonizes established human breast cancer xenografts (GI-101A) inducing their complete regression,[2] in comparison to nonresponding HT-29 colorectal carcinomas. We explored human cancer cell/VACV interactions in vitro and xenograft/VACV/host interactions in vivo adopting organism-specific expression arrays. Indeed, tumor rejection was associated in vivo with activation of interferon-stimulated genes (particularly interferon-γ induced) and immune effector functions as predicted in the ICR theory. As expected, the expression of CXCL9-11, CXCL12, CCL5 chemokines, Irf1, granzyme A and B, perforin, and FAS that compose the ICR was highly predictive of tumor rejection in this xenograft (Table 1).

This study provides the first prospective validation of a universal mechanism associated with tissue-specific destruction observable across species. Thus, ICR represents a tissue-specific targeting through immune enhancement for the therapy of cancer, chronic viral infections, and immune suppression in the context of allograft rejection or autoimmunity.

References:

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NEW AGENTS

Biochemical and Immunomodulatory Properties From Concholepas Hemocyanin (CCH) and Their Isolated Subunits

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Mollusk hemocyanins are considered among the most powerful immunogens in nature. Nowadays, biomedical applications employ hemocyanins as carrier in vaccines against pathogens and cancer, as adjuvant to pulse antigen presenting dendritic cells with tumor lysates, and as immunostimulant in the therapy of superficial bladder carcinoma. Curiously enough, the hemocyanin known as keyhole limpet hemocyanin (KLH) from Melanoides crenulata has been used for over 30 years for the above purposes, and it was not known whether other hemocyanins might be as efficient as an immunomodulatory agent. More recently, the hemocyanin from Concholepas concholepas has proved to be a reliable alternative to KLH. Interestingly, both hemocyanins differ not only in their origin but also in its organization. Although they have 2 subunits, a closer analysis showed that they display unique differences. In spite of it, both hemocyanins immunomodulate in a similar way as mammals' immune response: they increased natural killer cell activity with a concomitant T_H1 cytokine profile.

Our long-term goal is to know the relationship between the outstanding structural features of hemocyanins and their intrinsic immunomodulatory effects. We proposed that the identification of these structural features is essential to understand the properties of each part of hemocyanins. Here, biochemical and immunogenic properties of the CCHA and CCHB subunits from CCH were determined.

Homogeneous monomers of the CCH were obtained by anionic exchange chromatography and its purity was assessed by different methods. The study by surface-enhanced Raman spectroscopy provides evidence that CCH subunits display structural differences. Also, they differ in the content of carbohydrates: CCHA had a 3.6% w/w sugar with both N-linked and O-linked moieties, CCHB had a 2.5% w/w sugar with N-linked, while O-linked were nearly absent. Surprisingly, both subunits induced a similar humoral immune response, and showed an antitumor effect in a mice bladder cancer model without additional adjuvant. However, CCHA showed better efficiency when all the parameters analyzed were compared with CCHB. Therefore, our study supports the notion that CCHA monomer accounts for the most important features involved in the immunomodulatory properties of CCH, such as increased hydrophilicity, which could augment the humoral response, and higher content and diversity of carbohydrates that could enhance cellular response. Our results raise the potential therapeutic application of CCHA subunits on antitumor therapy since it is soluble and homogeneous, while CCH and KLH are heterogeneous.

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Functional Modulation of Dendritic Cells by Milatuzumab, a Humanized Anti-CD74 Monoclonal Antibody

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Milatuzumab (hLL1), a humanized anti-CD74 immunoglobulin G monoclonal antibody (MAb), has been shown to have therapeutic activity against CD74-expressing B-cell malignancies in vitro and in xenograft models. However, it is unclear whether this MAb has any effects on human antigen-presenting cells that normally express CD74. Here we investigated the functional modulation of dendritic cells (DCs), the professional and most potent antigen-presenting cells, exposed to milatuzumab. We found that at the concentration range from 0.05 to 5 μg/mL, milatuzumab up-regulated the expression of the antigen-presenting molecule, HLA-DR, and costimulatory molecules, CD54 and CD86, in human monocyte-derived DCs in a dose-dependent manner, suggesting that milatuzumab can enhance DC constitutive maturation. Although this effect was not reflected by an enhanced T-cell expansion, milatuzumab-treated DCs significantly promoted the differentiation of CD4+ naive T cells toward more T_H1 effector cells, and fewer T_H2 cells and nonpolarized T cells, suggesting that milatuzumab can modulate DC functions, inducing the polarization and differentiation of functional T_H cells. Unlike the cytotoxic response of B-cell malignancies to milatuzumab, immature DCs treated with this anti-CD74 MAb did not show apoptosis, even though they expressed high levels of CD74, with which milatuzumab bound efficiently. These data highlight the prospects of milatuzumab as a novel immunotherapeutic agent that possesses not only direct antiproliferative effects against CD74+ hematological malignancies, but also regulatory effects on DC-mediated immune functions.

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Phase 1, Randomized, Double-blind, Placebo-controlled, Single-dose, Dose-escalation Study Of Imprime PGG Injection (Imprime PGG) in Healthy Subjects

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Imprime PGG is a β 1,3/1,6 glucose polymer immunomodulator being developed for the treatment of cancer. Preclinical in vivo tumor model studies demonstrate neutrophil-mediated antitumor activity and survival enhancement when Imprime PGG is administered in combination with complement-activating monoclonal antibodies.

In a phase 1 study, 24 healthy subjects (subs) received a single intravenous infusion of placebo or Imprime PGG at doses of 0.5, 1.0, 2.0, 4.0, or 6.0 mg/kg in separate cohorts. In each cohort, 3 subs were randomized to Imprime PGG and 1 to placebo; safety in the previous cohort was determined before dose escalation. Subs were monitored for adverse events (AEs) as well as, physical examination, vital sign, electrocardiogram, and clinical laboratory results, with last evaluation at 7 days after dosing. Serum Imprime PGG levels were determined by an enzyme-linked immunosorbent assay. Pharmacokinetic parameters were analyzed using standard noncompartmental methods.

In total, 15/24 (62.5%) subs experienced AEs, 12/18 (66.7%) Imprime PGG subs, and 3/6 (50.0%) placebo subs. The most common AEs were headache (4 subs; 16.7%) and dyspnea (3 subs; 12.5%). There were no deaths, and none of the AEs were serious, severe in intensity, or clustered in a particular system organ class. Four subs (16.7%) prematurely discontinued Imprime PGG infusion because of AEs. Original plans maintained a 1-hour infusion at all dose levels. Following infusion-related AEs at 4.0 mg/kg, the infusion plan was revised, limiting dosing to 2.5 mg/min (at a concentration of 0.6 mg/mL); this resulted in longer infusion times for the 4.0 and 6.0 mg/kg cohorts. Four additional subs dosed at 4.0 mg/kg over 2 hours experienced no infusion-related AEs.

Study drug was also generally well tolerated in subs given 6 mg/kg over 3 hours. Treatment-related (TR) AEs were not reported in placebo subs but were reported in 9/18 (50%) Imprime PGG subs. An increase in the number of TR AEs from 0 in the 4.0 mg/kg (2-h infusion) cohort to 8 in the 6.0 mg/kg cohort, suggested that 6 mg/kg may be approaching a maximum-tolerated dose under the conditions administered; the number of TR AEs in the 0.5, 1.0, and 2.0 mg/kg cohorts were 2, 1, and 1, respectively. No clinically significant changes from baseline were observed at end of study for physical examination, vital signs, electrocardiogram, or clinical laboratories. Pharmacokinetic area under the curve assessments indicated that Imprime PGG concentration was linear over the doses administered. Mean $T_{\frac{1}{2}}\beta$ values ranged from 23.36 to 32.75 hours. Overall, after adjusting infusion rates, Imprime PGG was safe and well tolerated at single doses up to 6.0 mg/kg.

Phase 1, Randomized, Double-blind, Placebo-controlled, Multiple-dose, Dose-escalation Study Of Imprime PGGInjection (Imprime PGG) in Healthy Subjects

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Imprime PGG is a β 1,3/1,6 glucose polymer immunomodulator being developed for the treatment of cancer. Preclinical in vivo tumor model studies demonstrate neutrophil-mediated antitumor activity and survival enhancement when Imprime PGG is administered in combination with complement-activating monoclonal antibodies.

In a phase 1 study, 12 healthy subjects (subs) received 7 consecutive daily intravenous infusions of placebo or Imprime PGG at 1.0, 2.0, or 4.0 mg/kg in separate cohorts. Doses of 1.0 and 2.0 mg/kg were infused over 1 hour and 4 mg/kg over 2 hours. In each cohort, 3 subs were randomized to Imprime PGG and 1 to placebo; safety in the previous cohort was determined before dose escalation. Subs were monitored for adverse events (AEs), as well as physical examination, vital signs, electrocardiogram, and clinical laboratory results, with last evaluation on Day 30. Serum Imprime PGG levels were determined by an enzyme-linked immunosorbent assay. Pharmacokinetic parameters were analyzed using standard noncompartmental methods.

In total, 8/12 (66.7%) subs experienced AEs, 6/9 (66.7%) Imprime PGG subs, and 2/3 (66.7%) placebo subs. The most common AE was headache (2 subs; 16.7%). There were no deaths, and none of the AEs were serious, severe in intensity, or clustered in a particular system organ class. One placebo sub and one 4.0-mg/kg sub prematurely discontinued the study because of moderate AEs. Treatment-related AEs were reported in 1/3 (33.3%) placebo subs and in 2/9 (22.2%) Imprime PGG subs, both dosed at 4.0 mg/kg. Compared with 1.0 or 2.0 mg/kg Imprime PGG, the occurrence of AEs at 4 mg/kg (one which resulted in discontinuation), suggested that 4.0 mg/kg may be approaching a maximum-tolerated dose under the conditions administered. Some minor deviations from normal ranges were observed for clinical measures, but were not assessed as Imprime PGG related. One exception was a transient, dose-dependent increase in total neutrophil counts, which was considered a pharmacodynamic activity. There were no physical examination or electrocardiogram results considered AEs. Pharmacokinetic parameters were assessed on Day 0 (final draw 24 h after first dosing) and on Day 6 (final draw on Day 30, 24 d after final dosing). Steady state was attained at Day 6. Area under the curve Imprime PGG concentrations were linear over the doses administered at both evaluation times. Comparison of Day 0 versus Days 6 to 30 area under the curve (0–last) parameters revealed larger values at Day 6 to 30 for all treatment groups; $T_{\frac{1}{2}}\beta$ values also tended to be larger at Days 6 to 30 versus Day 0. Overall Imprime PGG was safe and well tolerated at multiple doses up to 4.0 mg/kg.

Phase I Study of BMS-663513, a Fully Human Anti-CD137 Agonist Monoclonal Antibody, in Patients (Pts) With Advanced Cancer (CA)

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Background: CD137 (4-1BB) belongs to the TNFR family and serves as a costimulatory molecule on T cells and other immune cells. In preclinical studies, anti-CD137 agonist antibody enhanced interferon-γ production and cytolytic activity and increased survival. We conducted a first-in-human phase I dose-escalation study of BMS-663513, a fully human anti-CD137 agonist monoclonal antibody in patients (pts) with cancer (CA).

Methods: BMS-663513 was administered intravenously every 3 weeks. In Part 1, the dose was escalated (0.3, 1, 3, 6, 10, and 15 mg/kg) in cohorts of 3 to 6 pts. In Part 2, pts with melanoma, renal cell cancer (RCC), and ovarian CA, 30 per arm, were stratified by tumor type and randomized to receive 1, 3, or 10 mg/kg dose levels. Tumor response (modified WHO) was determined after the fourth dose and then every 2 doses. After 4 doses, treatment continued in pts with antitumor activity and without dose-limiting toxicity. Peripheral blood samples were obtained at select times for exploratory biomarker analyses.

Results: A total of 115 pts (54 melanoma, 30 RCC, 30 ovarian, and 1 prostate) were treated. In Part 1, single dose-limiting toxicities were reported in the 0.3 mg/kg (Grade 3 neutropenia) and 15 mg/kg (Grade 4 neutropenia) cohorts. Overall, fatigue (all, 23%, Grade 3-4, 3%), reversible Grade 3-4 AST/ALT elevations (14%) and Grade 3-4 neutropenia (5%) were the most common agent-related adverse events. In evaluable subjects, 4/47 (8.5%) partial responses occurred in the melanoma cohorts, whereas durable stable disease ($\geq$ 6 mo) was noted in 4/47 (8.5%), 4/24 (16%), and 1/22 (4%) pts with melanoma, RCC, and ovarian CA, respectively. Interferon-inducible genes, serum neopterin levels, and activated T cells increased following a single treatment. No clear dose-response correlation was observed in any of these biomarkers.

Conclusions: BMS-663513 was tolerable across a wide dose range (0.3 to 15 mg/kg). As a single agent, BMS-663513 demonstrates clinical activity that justifies its further evaluation both as a single agent and in combination with other modalities.

Characterization of a Humanized Anti-HGITR Monoclonal Antibody (Mab), TRX518

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Glucocorticoid-induced tumor necrosis factor receptor (GITR) is expressed on a number of cell types including regulatory and effector T cells, B cells, natural killer (NK) cells and antigen-presenting cells. In murine studies, we and others have demonstrated that agonistic anti-GITR antibodies abrogate regulatory T cell suppressive effects and have potent adjuvant and anticancer properties. In the in vivo models, anti-mGITR Mab administration has been shown to (1) directly induce tumor immunity, (2) synergize with anti-CTLA-4 blockade, (3) augment cancer immunity in combination with vaccines, and (4) augment tumor immunity in combination with chemotherapeutic agents. Chimeric Mabs based on our parental murine 6C8 anti-hGITR Mab were constructed and demonstrated adjuvant effects in cynomolgus macaques. This abstract details the initial characterization of a fully humanized IgG1 monoclonal antibody that binds to the extracellular domain of hGITR, TRX518. A GMP cell line was generated and TRX518 was characterized in numerous in vitro assays. In addition to humanization, asparagine 297 of the heavy chain was substituted with alanine to eliminate the N-linked glycosylation site. This mutation abrogates Fc region functionality including antibody-dependent cellular cytotoxicity and complement-mediated lysis. Additionally, an asparagine residue in the second complementary-determining region was substituted to glutamine to eliminate a putative N-linked

glycosylation site. This mutation had no significant effect on the affinity to hGITR. TRX518 has a KD in the 10-9-10-10 M range, as measured by a Biacore assay using soluble hGITR-Fc as the immobilized molecule. TRX518 blocks the binding of GITR ligand (GITRL) to GITR in a competition enzyme-linked immunosorbent assay. TRX518 binds to GITR on CD4+ and CD8+ naive and memory T cells, B cells, NK cells, NKT cells, monocytes, and macrophages consistent to what has been described. TRX518 is co-stimulatory with plate-bound low dose anti-CD3 using peripheral blood lymphocytes (PBLs) in a tritiated thymidine uptake assay. TRX518 does not block an allogeneic mixed lymphocyte reaction nor does it induce apoptosis of PBLs, antibody-dependent cellular cytotoxicity against HUT78 cells stimulated with anti-CD3, or complement-mediated lysis of human PBLs. Plate-bound TRX518 that was air dried to reveal any putative superagonist effects did not induce appreciable cytokine release [interleukin (IL)-2, IL-6, IL-10, tumor necrosis factor-α, and interferon-γ] compared to that observed with a nonbinding isotype control antibody. Other studies to further define its mechanism of action are in progress. These data support the continued development of TRX518 for potential indications such as hematologic and solid tumors, chronic viral infections, and as a vaccine adjuvant.

Targeting Protein Tyrosine Phosphatases to Enhance Immune Targeting Against Receptor Tyrosine Kinase-overexpressing Cancers

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Overexpression of receptor tyrosine kinases (RTKs), such as EGFR, her2/neu, and EphA2, is a common observation in cancer, including head and neck cancer. Activated RTK signaling promotes cell proliferation, survival and migration/metastasis, thereby supporting oncogenic pathways. RTKs are typically phosphorylated upon activation, making them targets for ubiquitination and subsequent degradation mediated by the proteasome. This process may be functionally antagonized by the activity(ies) of 1 or more protein tyrosine phosphatases (PTPs). We hypothesized that by depleting PTP expression by specific gene knockdown in tumor cells that we may facilitate the proteasomal degradation of RTKs and that as a consequence, the frequency of RTK-derived epitopes that are presented in the context of major histocompatibility complex class I to cytotoxic CD8+ T cells would be enhanced, allowing even T-cell effector cells of modest functional avidity to now recognize and kill the treated cancer cells. SiRNA blockade of various PTPs caused a detectable reduction in the expression of multiple cancer-associated RTK proteins as measured by Western blot in some but not all cancer cell lines examined. However, conditional knock down of specific PTPs appeared to enhance tumor cell presentation of RTK-derived epitopes using RTK-specific CD8+ T lines. This suggests that changes in RTK expression levels which are below the current limits of detection may alter RTK processing into the proteasome sufficiently to allow for improved immune targeting of the treated cancer cells.

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TH-17, CYTOKINES, AND T CELL SUBSETS

Critical Role of TNF-α in the Expansion of Regulatory T Cells and Age Related Toxicity Following Immunotherapy

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Regulatory T cells (Tregs) are of particular interest with respect to cancer immunotherapy, due to their potential to suppress antitumor responses. In previous studies, we reported that potent immunotherapeutic regimens such as IL-2/IL-12, anti-CD40/IL-2, anti-CD40/IL-15 and CpG/IL-12 resulted in increased tumor survival but was accompanied by a concurrent marked expansion of Treg cells. This Treg cell expansion may be counterproductive to the therapeutic efficacy as the ratio of Treg to conventional T cells was greatly altered despite initial antitumor effects. Another problem with many immunotherapeutic regimens is a dose-limiting toxicity, which is problematic in young mice but even more severe in middle aged and aged mice. This toxicity is believed to be due to the induction of proinflammatory cytokines such as tumor necrosis factor (TNF)-α. Previous reports have suggested a role of TNF-α signaling in the expansion of Treg cells in vitro. However data on Treg cell expansion due to TNF-α secretion following immunotherapy has been lacking. We also observed significant amounts of TNF-α in the serum following immunotherapy. Therefore, we compared the effect of potent systemic immunotherapy in wild type (WT) and mice lacking TNF-α (TNF-α−/−) to determine how this cytokine affects the expansion of Treg cells in vivo. The treatment of both TNF-α−/− and WT mice with immunotherapy resulted in comparable expansion of CD4+ and CD8+ T cells. However, contrary to the findings in WT animals, when we treated TNF-α−/− mice, Treg cells were not expanded in the spleen or the lymph node. Interestingly, treatment of TNF-α−/− mice with immunotherapy did not result in signs of toxicity. Treatment of TNF-α−/− mice ranging in age from 3 to 16 months also did not result in gut or liver pathology as was observed in the WT animals. Due to these findings, current studies are evaluating antitumor efficacy in this model. Taken together these data suggest that TNF-α secretion following immunotherapy in vivo is critical for the expansion of Treg cells and furthermore plays a role in toxicity related to systemic immunotherapy. Thus, blockade of TNF-α in conjunction with systemic immunotherapy may promote enhanced therapeutic efficacy through improved compliance and heightened antitumor activity.

Sensitivity to Apoptosis of the CD8+CD45RA+CCR7− T-Cell Subset in the Blood Discriminates Cancer Patients From Healthy Controls

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Objective: We previously showed that CD8+ CCR7− T cells are more sensitive to spontaneous apoptosis than their CCR7+ counterparts. Patients with head and neck squamous cell carcinoma (HNSCC) have reduced numbers of circulating CD8+CCR7+ T cells relative to normal controls (NC). The functional and clinical significance of this phenotypic imbalance in HNSCC patients was investigated.

Methods: Expression of CCR7, CD45RA, CD28, Fas, proapoptotic, and antiapoptotic proteins of the Bcl-2 family and Annexin V (ANX) binding were evaluated by multiparameter flow cytometry in T cells of 22 patients with active disease, 29 with no evident disease, and 32 NC. Clinicopathologic and immunologic data were analyzed by multivariant statistics and hierarchical clustering. Heat maps relating sets of variables were analyzed by Spearman correlations.

Results: In patients, decreases in percentage and absolute numbers of CD8+CCR7+ cells in the periphery versus NC ($P < 0.001$) were accompanied by an expansion of CD8+CDRA+CCR7− terminally differentiated effector T cells. In contrast, CD8+CD45RA-CCR7− memory effector cells remained unchanged relative to NC. Despite their high frequency in patients, the CD8+CD45RA+CCR7− cells showed the highest ANX binding. Naive CD45RA+CCR7+ and central memory CD45RA−CCR7− cells showed minimal ANX binding. CCR7 expression on T cells was significantly associated with the T stage and type of treatment, but not with disease status (active disease vs. no evident disease), differentiation or tumor site. Hierarchical clustering analysis of T-cell subpopulations revealed 2 distinct clusters characterized by high ANX binding (patients) or low ANX binding (NC). The high frequency of early apoptosis (ie, ANX binding) within the CD8+CD45RA+CCR7− T cells was sufficient to discriminate between NC and patients with 94% accuracy. A heat map analysis of T-cell subsets confirmed a positive correlation between ANX binding and the absence of CCR7 on CD8+ T cells and a negative correlation between ANX binding and expression of CCR7 on these cells. In patients, the sensitivity of CD8+CCR7− cells to apoptosis was related to higher Bax/Bcl-2 and Bim/Mcl-1 ratios as well as higher Fas and lower FLIP expression than those in CCR7+ cells. Treatment of primary CD8+CCR7+ cells with the CCR7 ligand, CCL21, or survival cytokines, interleukin (IL)-2, IL-7, IL-15, resulted in a significant decrease in apoptosis and to normalization of the Bax/Bcl-2 and Bim/Mcl-1 ratios.

Conclusions: The rapid differentiation of naive to effector T cells accompanied by the loss of CCR7 and concomitant apoptosis of CD8+CCR7− cells in cancer patients compromise their antitumor responses and might serve as a disease biomarker in HNSCC.

Amplified and Targeted IL-15 Expression Vector for Cancer Immuno-gene Therapy

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Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a key role in regulating both innate and adaptive immune responses. It promotes the survival, proliferation, activation, and maintenance of natural killer cells and CD8+ T cells, also stimulates the function of neutrophils, macrophages, and dendritic cells. Therefore, IL-15 could be a potential cytokine for cancer immune therapy. The therapeutic effects of cytokine relate to its expression levels. Here we report amplified IL-15 expression plasmid vector pHi2-spIL15-CMV-tat and carcinoembryonic antigen (CEA)-positive tumor-specific amplified IL-15 expression plasmid vector pHi2-spIL15-CEA-tat. In pHi2-spIL15-CMV-tat, the IL-15 expression is driven by HIV2 LTR, which transactivated by CMV promoter controlled expression of HIV tat. In addition, the native IL-15 signal peptide is replaced by IL-2 signal peptide to enhance its secretion. Significant amount of IL-15 expression is achieved when transfected into tumor cells in vitro. In order to target IL-15 expression to CEA-positive cells, CEA promoter positively controlled IL-15 expression plasmid vector pHi2-spIL-15-CEA-tat were constructed by replacing the CMV promoter with CEA promoter, efficient and targeted IL-15 expression were achieved in CEA-positive tumor cells by pHi2-spIL15-CEA-tat. IL-15 contained in the supernatant of transfected cells stimulated spleen cell proliferation. Injection of IL-15 expression plasmids into the abdomen increased the survival of mice inoculated intraperitoneally with CT-20 tumor.

The Antitumor Effects of COX-2 Inhibitors in Tumor Microenvironment are Mediated by the Inhibition of TH17 Cells

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Background: Th17 cells represent a unique subset of CD4 T cells that produce the proinflammatory cytokine interleukin (IL)-17. Th17 cells play an active role in inflammation and autoimmune diseases. The nature and regulation of Th17 in the context of tumor immunity remain unknown. However, according to recent studies, Th17 cells have immune tolerogenic properties in tumor microenvironment. Cyclooxygenase-2 (COX-2) and its metabolite, prostaglandin (PG) E_2 affect tumorigenesis, including angiogenesis, invasion, and tumor-induced immune suppression. Their overexpression is association with inflammation and impaired immune cell function in many tumors. Therefore, we investigated the effects of COX-2 inhibitors on Th17 cells activation in tumor cells.

Methods: Lewis lung cancer cells (3LL) were inoculated into C57Bl/6 mice. Twenty mice were randomized into normal controls, 3LL inoculated controls, and celecoxib 10 or 100 mg/kg/d treated 3LL inoculated mice. The mice were sacrificed on day 28. The tumor mass of 3LL inoculated control mice was removed for isolation of tumor infiltrating lymphocytes (TIL). The isolated TIL were treated with PGE_2 and various concentrations of COX-2 inhibitor (PTPBS; 5, 15, and 30 μM) for IL-17 enzyme-linked immunosorbent assay. In addition, we assessed Western blotting for transcription factor retinoic acid-related orphan receptor (ROR)-γ and p-STAT3 that were recently described to be essential for differentiation of Th17 cells. For the FACS analysis, splenocytes that were isolated from the mice of each group were stained with CD4 FITC and IL-17 PE monoclonal antibodies.

Results: We assessed the effects of PTPBS on IL-17 production and found that the IL-17 production of TIL was increased with PGE_2 and decreased with PTPBS treatment, dose-dependent manner. The expressions of ROR-γ and p-STAT3 were increased in PGE_2-treated TIL; however, they decreased with PTPBS treatment. The FACS analysis demonstrated a decline in the percentage of CD4+IL-17+ in the celecoxib treated groups. A significant inhibition of tumor growth was observed in the groups of mice treated with celecoxib compared to the untreated 3LL inoculated control group.

Conclusions: These results of this study show that COX-2 inhibitor reduced IL-17 production, as well as ROR-γ and p-STAT2 expression in TIL. The percentage of Th17 was attenuated and ultimately decreased the tumor burden with celecoxib treatment. These findings suggest that the COX-2 inhibitor promotes antitumor reactivity through the inhibition of Th17 cells.

Gene Expression Profiling Signatures Associated With RCC Response to IL-2 Therapy

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Background: High-dose interleukin (IL)-2 therapy produces tumor responses in 15% to 23% of patients (pts) with metastatic renal cell cancer with 10% of patients experience durable responses. Treatment is associated with significant toxicity, and several other less toxic therapies are now available, making efforts to identify pts and tumors most likely to benefit from IL-2 a high priority. We attempted to identify gene expression profiles in tumor tissue predictive of response to IL-2.

Methods: Frozen tumor tissue was obtained pre–IL-2 treatment, total RNA was extracted and hybridized to Affymetrix U133 2.0 GeneChips. The samples were split into a training set (BIDMC) and an independent validation set (Dartmouth). In depth, bioinformatics analysis using various unsupervised and supervised learning mechanisms identified a set of genes differentially expressed between responders and nonresponders in the training set. A 6-gene "IL-2 Response Signature" was applied to the independent validation set to predict response to IL-2.

Results: The initial training set included 15 pts subsequently treated with HD IL-2 (6 responders). The 6-gene IL-2 Response Signature separated responders from nonresponders in the training set with 100% accuracy. Application of this predictive gene signature to the independent validation set (14 tumors from pts treated at Dartmouth with a DC vaccine+IL-2/IFN including 6 responders) confirmed the accuracy of predicting IL-2 response with 83.3% sensitivity and 100% specificity. Evaluation identified HLA-DQ α 1 as highly expressed in IL-2 responsive tumors in both pt sets, whereas HLA-DRB4 expression was diminished.

Conclusions: We have identified and validated a tumor-associated gene expression signature that appears to be associated with response to IL-2 therapy. Furthermore, the presence of immune recognition genes in this signature suggests that these findings may shed light on the biology of renal cell cancer responsiveness to IL-2 and possibly immunotherapy, in general.

Overexpression of CD39 and CD73 and the Lack of CD26 in Human Regulatory T Cells Lead to Endogenous Adenosine Production and Induce Immunosuppression

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Objective: Regulatory T cells (Treg) are responsible for the maintenance of self-tolerance, tolerance to tissue grafts, prevention of autoimmune diseases, and attenuation of antitumor responses in cancer patients by inhibiting functions of effector T cells. In this study, the involvement of the adenosinergic pathway in Treg-mediated immunosuppression was investigated. The ectonucleotidases, CD39 and CD73, convert ATP into adenosine by sequential dephosporylation. Adenosine, binding to the A2a receptor on effector T cells, suppresses T-cell functions. Adenosine

is metabolized by adenosine deaminase that is associated with the extracellular domain of dipeptidyl peptidase IV (CD26).
Methods: Expression of CD39, CD73, and CD26 on human CD4+CD25high Foxp3+ Treg isolated from peripheral blood mononuclear cells of 15 normal donors was evaluated by multiparameter flow cytometry and immunohistochemistry. Suppression mediated by sorted CD4+CD39+ and CD4+CD26– cells was assessed in CFSE-based suppression assays with autologous CD4+CD25– responder cells. ATP hydrolysis was measured using the luciferase-based ATP detection assay system. Adenosine in cell supernatants was analyzed by mass spectrometry.
Results: Expression of CD39 on Treg was higher ($P < 0.05$) compared to CD4+CD25– cells (79% ± 15 vs. 5% ± 2), while that of CD26 was lower ($P < 0.05$) in Treg compared to CD4+CD25– cells (25% ± 11 vs. 81% ± 11). Immunohistochemistry showed that CD73 was exclusively expressed by Treg. Sorted CD4+CD39+ cells and CD4+CD26– cells suppressed the proliferation of CFSE-labeled CD4+CD25neg responder cells in 5-day cultures. Sorted CD4+CD25+ cells hydrolyzed significantly greater levels of exogenous ATP and produced more of adenosine ($P < 0.05$) than CD4+CD25– cells. ARL67156, an ectonucleotidase inhibitor, decreased ($P < 0.05$) the suppression of proliferating responder cells (38% ± 9% vs. 19% ± 1%). The inhibitory effects of CD4+CD25+ cells on the proliferation of CD4+CD25– cells were mimicked by 2-chloroadenosine, a stable adenosine analog. ZM241385, a selective A2a receptor antagonist, reduced the suppressive effects ($P < 0.05$) of CD4+CD39+ cells from 36% ± 1% to 9% ± 1.
Conclusions: Expression of CD39 and CD73 characterizes human Treg, while CD26 is not expressed on Treg. The ability to hydrolyze ATP and consequently produce adenosine is one of the mechanisms used by human Treg to mediate suppression of effector T cells via A2a receptor signaling.

High Dose Continuous Infusion + Pulse Interleukin-2 With Famotidine has Activity in Metastatic Kidney Cancer

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Interleukin-2 (IL-2) given via continuous intravenous infusion elicits lymphokine-activated killer cell (LAK) cytotoxicity against kidney cancer cells. Hank reported that LAK subsequently pulsed with additional IL-2 show increased cytotoxicity against tumor cells in vitro. Famotidine may increase lymphocyte killing of cancer cell lines by increasing IL-2 uptake at the IL-2 receptor (Tsunoda). A preliminary report had suggested activity of the combination of continuous infusion plus pulse IL-2 with famotidine in kidney cancer (Quan, 2005). Fourteen patients received famotidine 20 mg intravenously twice per day and continuous infusion IL-2 (18 MIU/m^2/24 h) for 72 hours followed by a 24-hour rest, then pulse IL-2 at 18 MIU/m^2 over 15 to 30 minutes preceded by famotidine 20 mg intravenous. Cycles were repeated every 3 weeks. Patient characteristics included median age of 60 years (range, 29 to 74), 10 males, 7 with no prior therapy, 6 had undergone nephrectomy prior to appearance of metastases, median ECOG performance status = 1 (0 to 1). Most common metastatic sites were lymph node (9) and lung (8). Median number of cycles received was 6 (1 to 9). Most common toxicities were fever, hypophosphatemia, nausea/emesis, rigors, and hypomagnesemia. There were no treatment-related deaths. Four partial responses have been seen (29% total response rate). Responding sites have been lungs, lymph nodes, soft tissue, and kidney primary. Two patients have been rendered surgical complete responders and are alive without disease at 48.5+ and 36.25+ months. Median survival for all patients is 10.4 months. The combination of famotidine and high-dose continuous infusion + pulse IL-2 is active in metastatic kidney cancer.

Induction of CD56 Positive Lymphocytes by Pulse Interleukin-2 and Famotidine in Peripheral Blood of Patients With Melanoma or Kidney Cancer

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High-dose bolus or continuous infusion interleukin-2 (IL-2) schedules have been associated with increased lymphokine-activated killer cell (LAK) numbers and cytotoxic activity. LAKs are felt to be CD56 positive on flow cytometry. Daily intravenous doses of IL-2 of 18 to 21.6 MIU/m^2 over 15 to 30 minutes ("pulses") have been developed to attempt to lessen toxicity of this therapy. Previously, we have shown that patients with metastatic melanoma or kidney cancer may be treated safely with pulse IL-2 for 5 days preceded by famotidine 20 mg intravenously. Cycles were repeated every 21 days. Responses in both tumor types have been noted. Because LAK numbers have not previously been described with this regimen, we have examined CD56 numbers via peripheral blood flow cytometry in 11 patients with samples scheduled at baseline, after 2 cycles, and 2 months later/after 4 cycles. Eight patients had melanoma, 3 had kidney cancer.
Median CD56 counts after 2 cycles was significantly higher than baseline ($P = 0.001$). Similarly, CD56 counts 2 months later were also greater than baseline ($P = 0.009$). There was no difference between median values after 2 versus after 4 cycles. Patients who were clinical responders had median CD56 count = 650 after 2 cycles as compared to nonresponders who had median CD56 = 290 ($P = 0.005$).
CD56 counts are significantly elevated in patients treated with pulse IL-2 with famotidine and clinical responders have higher CD56 than nonresponders.
Median CD56 Counts (/mm^3)

Baseline	After 2 Cycles	After 4 Cycles
130	430	338

CD40 Dependent Induction of TH17 Effector Cells From T Regulatory Cells Using the Immune Modulator B7-DC XAB

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B7-DC XAb is a human IgM antibody isolated from the serum of a patient diagnosed with Waldenstrom's macroglobulinemia. The antibody binds to B7-DC/PD-L2 molecules on the surface of murine and human dendritic cells (DCs) and stimulates the DCs to become potent activators of naive T cells. Binding of pentameric B7-DC XAb results in cell surface cross-linking and activation of multiple signaling cascades in DCs downstream of an assembled molecular cap. Recently, we have documented that the co-culturing of CD25+T regulatory cells (Tregs) with the antigen-pulsed B7-DC Xab-treated DCs results in the conversion of Tregs into Th17 cells. The Treg conversion to T effector cells is dependent on interleukin (IL)-6. We have shown that cross-linking B7-DC on DCs leads to the activation of NFkB through the PI3K-Akt pathway and IL-6 secretion. Activation of nuclear factor kappa B (NFkB) leads to the protection of DCs against cell death upon cytokine withdrawal or upon induction of apoptosis by vitamin D3 analog. We have identified TREM-2 as one of the proteins recruited by B7-DC cross-linking. In the absence of TREM-2, B7-DC Xab-mediated induction of antigen uptake in the mature DCs is compromised. However, TREM-2 was not found to be necessary for the activation of NFkB or for the conversion of Tregs. Therefore, we sought to delineate the upstream molecules regulating the ability of activated DC to promote DC survival in response to apoptotic signals and to convert T regulatory cells into IL-17+ effectors. Here, we show by FRET and co-immunoprecipitation that CD40 is also recruited into the macromolecular cap. DCs that lack the expression of CD40 molecule did not activate Akt or NFkB in response to B7-DC XAb. Moreover, B7-DC XAb treatment failed to protect the DC from cytokine withdrawal or vitamin D3-induced cell death. The presence of CD40 is necessary for the secretion of IL-6 as CD40−/− DCs activated with B7-DC XAb do not secrete IL-6. The CD40-deficient DCs were unable to convert Tregs into IL-17+ effector T cells. Finally, the presence of CD40 on the DCs in vitro and in vivo is important for the generation of T effector cells capable of providing tumor protection against B16 melanoma or WEHI-3.

IL-7 and IL-15 Differentially Regulate CD8+ T Cell Subsets During Contraction of the Immune Response

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While it is known that interleukin (IL)-7 and IL-15 influence the survival and turnover of CD8+ T cells, less is known about how these cytokines affect different subsets of CD8+ T cells during the course of the immune response. We find that IL-7 and IL-15 differentially regulate CD8+ T-cell subsets defined by KLRG1 and CD127 expression during the contraction phase of the immune response. The provision of IL-15, or the related cytokine IL-2, during contraction, led to the preferential accumulation of KLRG1hiCD127lo CD8+ T cells, while provision of IL-7 instead favored the accumulation of KLRG1loCD127hi. Interestingly, while both IL-7 and IL-15 induced proliferation of KLRG1lo CD8+ T cells, KLRG1hi cells exhibited an extraordinarily high level of resistance to cytokine-driven proliferation in vivo despite their dramatic accumulation upon IL-15 administration. These results suggest that IL-15 and IL-2 greatly improve the survival of KLRG1hi CD8+ T cells, which are usually destined to perish during contraction, in the absence of proliferation. As the availability of IL-15 and IL-2 is increased during periods of extended inflammation, our results suggest a mechanism where a population of cytokine-dependent KLRG1hi CD8+ T cells is temporarily retained for improved immunity. Consideration of these findings may aid in the development of therapies for augmenting T-cell–directed immunotherapeutic strategies against cancer and infectious disease.

Serum Vascular Endothelial Growth Factor (VEGF) and Fibronectin as a Potential Predictor of Responsiveness to High-dose Interleukin (IL)-2 Therapy

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Systemic administration of high-dose human recombinant (hr) interleukin (IL)-2 induces durable therapeutic responses in a small subset of patients with metastatic melanoma and renal cell carcinoma. Simple pretreatment predictors of response, able to increase the therapeutic value of this agent, have not been identified.

In the present study, we prospectively collected sera from patients with metastatic melanoma or renal cell carcinoma for protein analysis using a customized multiplex antibody-targeted protein array platform that surveyed the expression of soluble factors associated with tumor immunobiology. A training set from 10 patients' samples identified 68 potentially relevant factors that were then tested in an independent validation set of 49 patients. Class comparison was then applied to identify biomarkers most closely associated with clinical response.

Class comparison revealed a cluster of 11 biomarkers significantly associated with therapeutic outcome. Vascular endothelial growth factor (VEGF) and fibronectin were identified as independent predictors of response (Fig. 1A). In particular, high dose of these proteins correlated with lack of clinical response and decreased clinical survival (Fig. 1B).

This study strongly supports the negative predictive value of pretreatment serum VEGF and fibronectin in patients with metastatic cancer undergoing hrIL-2 therapy. Moreover, the measurement of both proteins could be used to exclude patients unlikely to respond to IL-2 treatment.

CTLA-4 Blockade Increases Interferon-gamma–producing $CD4^+ICOS^{HI}$ Cells to Shift the Ratio of Effector to Regulatory T Cells in Cancer Patients

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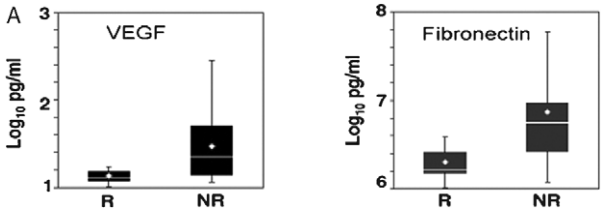

FIGURE 1A. VEGF and fibronectin protein levels from pretreatment sera in responding (R) and nonresponding (NR) IL-2–treated patients. Data are shown as median (central line), quartiles (boxes) and range (whiskers).

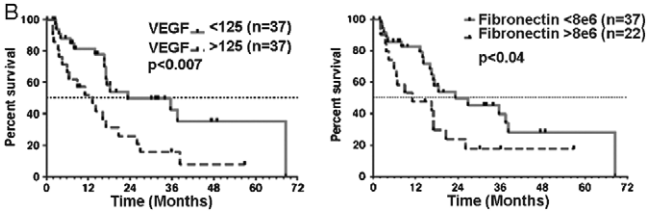

FIGURE 1B. Kaplan-Meier survival estimate of overall survival for IL-2–treated patients according to pretreatment serum VEGF level cut off 125 pg/mL ($P<0.007$) and fibronectin level cut off 8×106 pg/mL ($P<0.04$). IL indicates interleukin; VEGF, vascular endothelial growth factor.

The role of inducible co-stimulator (ICOS)-expressing T cells has been elusive. Studies in mice and in vitro experiments using human T cells have reported seemingly conflicting data, with some reports suggesting a regulatory function for ICOS-expressing T cells while others implicate them as effector cells. Here, we present data from cancer patients that establish ICOS-expressing CD4 T cells as effector T cells. ICOS expression and the ratio of effector to regulatory T cells are increased after blockade of cytotoxic lymphocyte antigen-4 (CTLA-4), a critical molecule for limiting T-cell activity. $CD4^+ICOS^{hi}$ T cells from treated patients have increased production of an immune-enhancing T_H1 cytokine interferon-γ, and can recognize the NY-ESO-1 tumor antigen. Thus, a major functional impact of CTLA-4 blockade is expansion of $CD4^+ICOS^{hi}$ T cells to play a role as interferon-γ–producing effector T cells.

Expansion of Functional CD4(+)CD25(High)FoxP3(+) Treg is Mediated by Tumor-derived Microvesicles

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Introduction: Tumor-derived microvesicles (MVs), which are present in the body fluids of cancer patients represent one mechanism of immune suppression and thus might contribute to tumor progression. Regulatory T cells (Tregs) expand in patients with cancer and inhibit anti-tumor immune responses. They play a major role in tumor escape from immunosurveillance. The goal of the study was to determine whether tumor-derived MVs contribute to human Treg expansion and suppression function.

Methods: MVs were isolated and purified from the supernatants of the following tumor cell lines: OVCAR3, SKOV3 (ovarian cancers), PCI-13 (oral cavity cancer), and from supernatants of immature DC (iDC) cultured from mononuclear cells of normal donors (n = 4) or patients with head and neck cancer (n = 5). CD4(+)CD25(high)FoxP3(+) and CD4(+)CD25(−) cells sorted by flow cytometry were cultured with MV (5 to 15 μg/mL) for different time periods (6, 12, 24 h). Proliferation of freshly isolated or rapamycin expanded Treg ± MV was determined by cell counts and in CFSE assays. Apoptosis of cultured cells ± MV was assessed by Annexin-V (ANX) binding. MV-sensitive CD8(+) Jurkat cells were used as positive controls for MV-mediated apoptosis.

Treg suppressor functions was tested in co-cultures with CFSE-labeled, OKT3-activated autologous CD4(+) CD25(−) responders cells. Multicolor flow cytometry was used to determine the phenotype of Treg ± MV. Activation of STAT3 in Treg was measured in Western blots. Culture supernatants were tested for levels of cytokines in Luminex-based assays.

Results: Tumor-derived MV or MVs derived from iDC enhanced the expansion (mean 8-fold) of Treg relative to control (–MV) cultures Treg were resistant to MV-mediated apoptosis relative to Jurkat cells, which were sensitive (5% vs. 65% ANX binding cells, respectively); ($P < 0.05$). Tumor-derived or iDC-derived MV enhanced the expression of HLA-DR, CTLA4, and intracellular perforin ($P < 0.05$) and increased STAT3 phosphorylation in Treg. Treg co-cultured with MV mediated stronger suppression ($P < 0.01$) of proliferation in autologous responder cells.

Conclusions: Our study suggests that MV released by human tumors or iDC can promote Treg expansion and their suppression function. This represents a newly defined mechanism that might be involved in regulating peripheral tolerance by tumors or immature dendritic cells.

Interleukin-15 Enhances NK-cell Cytotoxicity in Patients With Acute Myeloid Leukemia by Upregulating the Activating NK-cell Receptors

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Introduction: The triggering of the natural killer (NK)-cell cytotoxicity is determined by the balance of activating and inhibitory signals received via the broad repertoire of receptors found on individual NK cells. Interleukin-15 (IL-15) has a major role in NK-cell homeostasis. It regulates NK cells differentiation from hematopoietic progenitors, stimulates the expansion of peripheral NK cells and supports their survival. We hypothesize that IL-15 may also enhance antileukemic effects of autologous NK cells in patients with acute myeloid leukemia (AML).

Methods: The NK-cell receptor repertoire and NK activity were studied in 17 patients with newly diagnosed AML prior to any treatment and in 10 normal donors (NC). The ex vivo effects of IL-15 on the NK-cell receptor expression and cytotoxicity were also investigated.

Results: The expression of activating natural cytotoxicity receptors NKp30 and NKp46, and the C-type lectin receptors NKG2D and NKG2C was significantly decreased ($P < 0.001$ to 0.02) in the AML patients in NK-cell subsets compared to the NK-cell subsets in NC. In addition, NK cytotoxicity was lower ($P < 0.0003$) in the AML patients at diagnosis compared to that in NC. To assess the effect of IL-15 on NK-cell receptor expression, we cultured NK cells sorted from patients with AML at diagnosis in the presence of IL-15. A significant up-regulation of the NK-cell–activating receptors was observed compared to preculture levels in both NK-cell subsets (Table 1). Concomitant with the up-regulation of activating receptors following IL-15 stimulation, there was a significant increase in the NK-cell cytotoxicity against K562 cells targets and the patients' autologous leukemic blasts ($P < 0.05$). Addition of blocking antibodies to the activating receptors reduced the levels of the NK cytotoxicity.

Conclusions: IL-15 increases NK-cell lytic activity in patients with AML by up-regulating expression of the NK-cell activating receptors. These data support the use of IL-15 as a platform for autologous NK-cell based therapies in AML patients.

TRAFFICKING AND IN VIVO IMAGING

Evaluation of Angiogenesis Using Micro-computed Tomography in a Xenograft Mouse Model of Lung Cancer

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Rationale: Quantitative evaluation of lung tumor angiogenesis using immunohistochemical techniques has been limited by difficulties in generating reproducible data.

Experimental Design: To analyze intrapulmonary tumor angiogenesis, we used high-resolution micro-computed tomography (micro-CT) of lung tumors of mice inoculated with Lewis lung carcinoma (LLC1) or adenocarcinoma (A549) cell lines. The lung vasculature was filled with the radiopaque silicone rubber, Microfil, via the jugular vein (in vivo application) or pulmonary artery (ex vivo application).

Results: Quantitative analysis of lung tumor microvessels imaged with micro-CT showed that more vessels (mainly small, $< 0.02\,mm^2$) were filled using the in vivo (5.4%) compared with the ex vivo (2.1%) method. Furthermore, human adenocarcinoma lung tumor bearing mice treated site-specifically with bevacizumab (an antibody against vascular endothelial growth factor) showed significantly reduced lung tumor volume and lung tumor angiogenesis compared to untreated mice as assessed by micro-CT. Interestingly microvascularization mainly the smaller vessels ($< 0.02\,mm^2$) were reduced following bevacizumab treatment. This observation with micro-CT was nicely correlated with immunohistochemical measurement of microvessels.

Conclusions: Therefore, micro-CT is a novel method investigating tumor angiogenesis and might be considered as an additional and complementary tool for precise quantification of angiogenesis.

TUMOR ESCAPE/TUMOR MICROENVIRONMENT

Mechanism of Membrane-bound TGF-β1 Regulation in HNSCC Cell Lines

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Presence of cell membrane-bound transforming growth factor (TGF)-β1(mTGF-β1) has been reported recently in regulatory T cells but there have been only a few studies of that in cancer cells. In this study, we investigated the regulation of mTGF-β1 expression in 5 HNSCC cell

TABLE 1. The Effect of IL-15 on the Activating Receptor Expression in AML Patients

NK-cell Activating Receptors	CD56brightCD16– in AML Pts at Diagnosis % Positive	CD56brightCD16– in AML Pts After IL-15 Stimulation % Positive	*P*	CD56dimCD16+ in AML Pts at Diagnosis % Positive	CD56dimCD16+ in AML Pts After IL-15 Stimulation % Positive	*P*
NKp30	30	43	0.02	23	38	0.006
NKp46	29	47	0.003	33	45	0.001
NKG2C	30	40	0.005	24	32	0.006
NKG2D	44	63	0.005	39	62	0.007

AML indicates acute myeloid leukemia; IL, interleukin; NK, natural killer; Pts, patients.

lines using FACS analysis. Without TGF-β1 secretion, HNSCC cells induced phosphorylation of SMAD3 in immune cells upon direct cell-to-cell contact, in which mTGF-β1 was observed to be expressed in these cancer cell lines. Through blocking antibody and exogenous cytokine treatment experiments, we found that mTGF-β1 was induced by activated immune cells-derived factors such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α but not interleukin (IL)-1β, IL-6, and IL-2. In addition, IFN-γ and TNF-α was shown to have synergistic effect on mTGF-β1. Moreover, because exogenous TNF-α induced endogenous TNF-α mRNA expression, this TNF-α autocrine loop may be important. In contrast to previous reports on some immune cells, we confirmed that mTGF-β1 in this model was not of rebound form of once secreted TGF-β1, for low pH elution treatment did not decrease mTGF-β1 level and exogenous rhTGF-β1 did not increase mTGF-β1. Inhibitors of transcription (Actinomycin D), translation (cyclohexamide), or membrane-translocation (brefeldin A) effectively blocked induction of mTGF-β1, which led to our interpretation that induction of mTGF-β1 by IFN-γ and/or TNF-α occurs through de novo synthesis. These findings suggest that some cancer cells can detect immune activating cytokines such as IFN-γ and TNF-α and actively block antitumor immunity by induction of mTGF-β1.

A Novel Mechanism of Late Gene Silencing Driven SV40 Transformation of Human Mesothelial Cells

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Suppression of the late gene expression, usually by integration of the viral DNA into the host genome, is a critical step in DNA tumor virus carcinogenesis. Simian virus 40 (SV40) induces high rates of transformation in infected primary human mesothelial cells (S-HM) in tissue culture, leading to the formation of immortal cell lines (S-HML). The studies described here were designed to elucidate the unusual susceptibility of primary human mesothelial cells (HM) to SV40 carcinogenesis. We found that S-HML contained wild type, mostly episomal SV40 DNA. In these cells the early genes that code for the viral oncogenes are expressed, at the same time, the synthesis of the late genes capsid proteins is suppressed and S-HML are not lysed. Late genes suppression is achieved through the production of antisense RNA molecules. These antisense RNA molecules originate in the early region of the SV40 circular chromosome and proceed in antisense orientation into the late gene region, leading to the formation of highly unstable double strand RNA that is rapidly degraded. Our results reveal a novel biological mechanism responsible for the suppression of late viral gene products, an important step in viral carcinogenesis in humans (Figs. 1, 2).

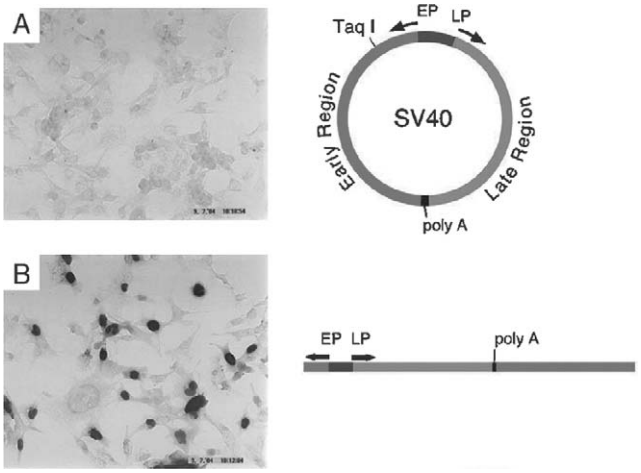

FIGURE 1. Linearized SV40 DNA rescues VP-1 synthesis in a fraction of transfected S-HML cells. 24 hour after transfection cells were assayed by immunocytochemistry. (A), lack of VP-1 expression in S-HML1 24 hour after electroporating 10 μg of circular SV40 DNA. The arrows on the SV40 map represent the direction of transcription from the early and late promoters (indicated as EP and LP). The region containing the early and late polyadenylation sites is indicated as poly A. (B), VP-1 immunostaining of S-HML1 24 hour after electroporating 10 μg of Taq I-digested SV40 DNA. Note VP-1 expression in these cells. As expected, reactivation is transient and disappears 3 to 5 days after transfection. Similar results were obtained in S-HML4.

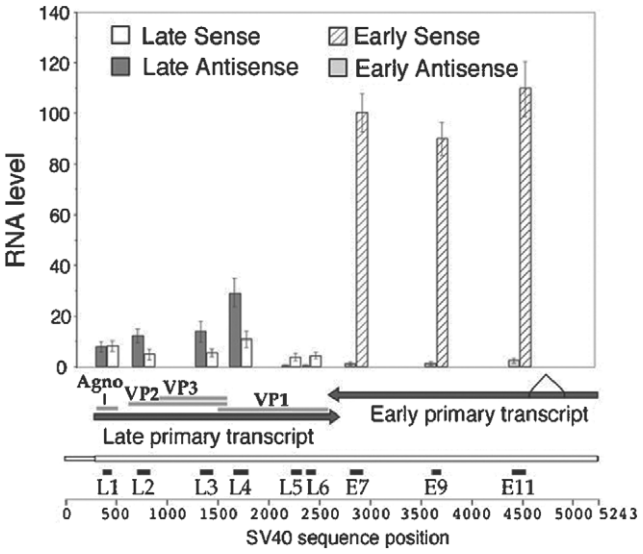

FIGURE 2. Transcriptional map of the SV40 genome in S-HML1 demonstrates the presence of late antisense transcripts. RNA was measured by strand-specific reverse transcriptase followed by qPCR. The PCR amplicons are reported below a schematic map of the SV40 genome in linear form (nucleotide numbering is from GenBank # AF316139). Average values (± SD) were calculated from four qPCR runs of samples derived from three independent strand-specific reverse transcriptase experiments. In the graph the average value of sense RNA from the early region (amplicons E7, E9 and E11) is set to 100. The amplified fragments from the early region are almost exclusively from ''sense'' RNA. Instead, comparable levels of both ''sense'' and ''antisense'' amplicons are generated from the late region.

Altered Gene Expression Patterns in Primary-versus-Metastatic Melanoma: Impact of Interactions With Stromal Cell Components

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High throughput gene expression profiling methods have contributed to a better understanding of the mechanism of tumor progression and metastasis in a variety of cancer types, including malignant melanoma. Most of these studies are performed using microdissected melanoma tissue samples from different patients. However, the amount of stromal components in each tumor sample as well as the heterogeneity between individuals can greatly influence the results obtained. It is well documented that malignant tumors of similar histology demonstrate great variation in their gene expression profiles in different patients, whereas profiles of primary/metastatic tumors derived from the same patient are more similar.

Therefore, comparing gene expression patterns of related paired samples of primary and metastatic melanoma cells can reveal subtle differences in their expression profile, thereby pinpointing the genes that are critical for the metastatic dissemination of malignant melanoma. Here, we characterized genes specific for the metastatic phenotype using melanoma cell lines derived from either primary cutaneous tumor or metastases from the same patients (2 patients, 5 cell lines); this approach identified 52 genes with significantly different expression in metastases. Some of these genes could be assigned to different pathways of cellular mechanisms and processes, revealing an important role for cellular growth and proliferation, signal transduction and gene expression. A number of these genes, such as, CXCR6, PAR2, and Neuroserpin have already been reported to be involved in metastasis in other cancer types or in melanoma, whereas the remainder represent new players, which may have an important role in melanoma metastasis. Gene expression profiling of melanoma cells and fibroblasts following interaction in mixed cultures revealed a greater susceptibility of fibroblasts to alterations in gene expression patterns caused by metastasis-derived melanoma cell lines, compared to their primary tumor-derived counterparts. Reciprocally, metastasis-derived melanoma lines proved to be more resistant to alterations induced by co-culture with fibroblasts compared to primary tumor-derived cell lines. This in vitro model may help further our understanding of melanoma development and metastasis and facilitate design of novel therapeutic interventions targeting the tumor cells themselves, or the supportive but genetically more stable tumor stroma. The model lends itself well to functional assays of antitumor T cells and to cultures under more physiological oxygen levels than usually employed (air).

CCL28 a New Link Between Hypoxia Angiogenesis and Tumor Immune Evasion

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Hypoxia is now recognized as one of the major contributors to cancer progression and to treatment failure. The precise role of hypoxia signaling in modulating the tumor microenvironment and cancer outcome still needs to be defined. In this work, we sought to understand the effect of hypoxia in immune regulation in the tumor microenvironment. We characterized the expression profile of genes implicated in immune response by real-time quantitative polymerase chain reaction low-density microarray in 17 human ovarian cancer cell lines in vitro. CCL28 was one of the most up-regulated genes identified in 9 out of 17 ovarian cancer cell lines. Migration assays with peripheral blood mononuclear cells using supernatants from hypoxic ovarian cancer lines showed a preferential migration of CD4+, CD25+ FoxP3+ T cells, suggesting a link of hypoxia to regulatory T cells (Tregs). Because we have previously shown that increased Treg infiltration is associated with short survival in ovarian cancer, we also explored the relationship between CCL28 expression and disease outcome. Results showed that survival for patients with high CCL28 expression was short in comparison with patients with low expression of CCL28. Next, to investigate the role of CCL28 in ovarian cancer in vivo, we transfected the well-characterized mouse ovarian cancer model ID8 with CCL28. ID8-CCL28 or wild-type ID8 cells were injected intraperitoneally into the C57Bl/6. Stable expression of CCL28 in ID8 tumor cells resulted in a faster tumor and ascites progression in comparison with the parental ID8 cells. Next, we characterized the cell infiltrate and cytokine profile of the ascites of animals injected with ID8-CCL28. In these animals, we found a higher number of CD4+, CD25+, FoxP3+ cells and a higher expression of interleukin-10, vascular endothelial growth factor, MCP-1, MCP-2, and MCP-3. To investigate the role of the CD4+, CD25+, FoxP3+ cells in the progression of ID8-CCL28 tumors in vivo, we depleted the CD25+ cells 4 days before the tumor challenge. CD25 depletion resulted in a partial decrease of the tumor growth suggesting a role of Tregs in the CCL28-mediated tumor progression. To our knowledge, these results provide the first evidence establishing a link between hypoxia and cancer immune evasion and could lead to alternative and more efficient therapeutic approaches.

Doxorubicin Selectively Down-regulates B7-H1 Surface Expression in Breast Cancer Cells

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B7-H1 is a T-cell inhibitory molecule expressed in many cancer types leading to immune escape of cancer cells. The expression of this molecule in high-risk breast cancer patients has been shown in our previous study. Breast cancer patients usually undergo neo-adjuvant chemotherapy (such as docetaxel and doxorubicin) before surgery. Breast cancer patients with estrogen receptor positive tumors undergoing such therapy showed a significantly less expression of B7-H1 compared with patients who did not receive the chemotherapy. To demonstrate this in vitro, we investigated the effect of docetaxel and doxorubicin on the down-regulation of B7-H1 in the MDA-MB-231 breast cancer cell line. Interestingly, doxorubicin was able to down-regulate the expression of B7-H1 in the MDA-MB-231 breast cancer cell line at clinically relevant concentrations compared to docetaxel, which had no effect. The effect of doxorubicin on B7-H1 down-regulation was independent of the drug effect on the cells growth or its induction of apoptosis. B7-H1 down-regulation was also mediated by other members of anthracyclines, such as daunorubicin and mitoxantrone. Our preliminary data indicates that doxorubicin might have an immunomodulatory function through down-regulation of B7-H1 molecule.

Human Activated T Lymphocytes Modulate Indoleamine 2,3-dioxygenase Expression in Tumors Through T_H1/T_H2 Balance

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Mechanisms of immune tolerance allowing tumor escape from immune surveillance can occur in the tumor microenvironment. These include local production of immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO). This interferon-gamma (IFN-γ)–inducible enzyme, produced by many primary human tumors, depletes tryptophan locally and exerts a suppressive effect on T lymphocyte functions. However, IDO induction mechanisms in tumor cells have not been characterized.

We observed IDO mRNA expression in 47% of breast and 75% of kidney cancer specimens. We also observed the presence of a variety of tumor-infiltrating immune cells in breast and kidney clinical samples, including T lymphocytes with a generally activated phenotype. Moreover, these infiltrating T cells were more prone to produce IFN-γ when compared with blood T lymphocytes. We therefore hypothesized that tumor cells express IDO in response to activated tumor-infiltrating lymphocytes.

To confirm our hypothesis, we co-cultured activated T cells with 3 breast and 2 kidney cancer cell lines. IDO mRNA expression was induced by activated CD4+ and CD8+ T cells in all tumor cell lines evaluated, while protein expression was confirmed in 3 tumor cell lines. This induction was partly driven by IFN-γ. Fascinatingly, interleukin-13, which is also produced by the same activated T cells, was shown to negatively modulate IFN-γ–induced IDO. As interleukin-13 is known to be in the breast cancer environment, T_H1/T_H2 cytokine balance provided by activated lymphocytes in the tumor microenvironment may have an impact on immunomodulatory mechanisms.

Expansion of Tumor Cells Deficient in CXCL9/MIG Production During Growth of Cutaneous Tumors

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Mechanisms that allow malignant tumors to expand because of escaping host immune surveillance remain poorly understood. Our recent studies have indicated high frequency of tumor cells deficient in production of CXCL9/Mig chemokine within advanced cutaneous murine MCA205 fibrosarcomas. These Mig-deficient tumor variants were highly tumorigenic and their growth in vivo was not suppressed by T cells, suggesting that these tumor cells arise and/or expand as a result of T-cell–mediated tumor immunoediting. To test this, tumors were retrieved from immunocompetent (wild type) or from T-cell–deficient (RAG1 KO) mice at early and late stages of tumor growth. Tumor cell suspensions were prepared by enzyme digestion of tumors and were subcloned to generate single tumor cell clones. These clones were tested for the ability to produce Mig after interferon-γ stimulation in vitro. We observed a sharp increase in the number of Mig-deficient tumor cells retrieved from the tumors growing in wild-type mice at the later stage of their growth. The frequency of these Mig-deficient tumor cells was at least 2 times higher in the tumors growing in wild-type mice than in the tumors growing in RAG1 KO mice. The results suggest the potential role of T cells in immune selection and outgrowth of Mig-deficient tumor variants during tumor growth. Lack of the ability to produce Mig may be an important mechanism employed by tumor cells to evade immune surveillance and this mechanism may contribute to rapid expansion of tumors that consist mainly of Mig-deficient variants.

Cell Surface Bound MUC16 (CA125) Shields Ovarian Tumor Cells From Natural Killer Cell Mediated Attack

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A majority of patients with epithelial ovarian cancer show an increase in serum levels of CA125. This antigen is a repeating peptide epitope expressed on MUC16, a 3 to 5 million Da mucin. MUC16 is overexpressed on the cell surface (csMUC16) and is also shed (sMUC16) by tumor cells following proteolytic cleavage. We previously reported that sMUC16 inhibits natural killer (NK) cell-mediated cytolysis of tumor targets. Here, we demonstrate that the ovarian tumor cell line OVCAR-3 specifically utilizes csMUC16 to evade NK-cell responses. NK cells target subsets of the ovarian cancer cell line OVCAR-3 that carries low amounts of csMUC16. csMUC16 knock-down results in approximately 2-fold higher susceptibility to NK-cell–mediated lysis even though these modified target cells display 10-fold to 20-fold higher levels of human leukocyte antigen class I antigens on their surface. Confocal microscopy experiments indicate that csMUC16 prevents conjugate formation and activating synapses between NK cells and cancer cells. csMUC16 also prevents lysis of tumor cells by NK cell leukemia cell line, NKL. Cancer cells that are resistant to NKL-mediated lysis express higher levels of csMUC16 than unchallenged cells. These results indicate that csMUC16 prevents efficient recognition of epithelial ovarian cancer cells by NK and potentially other immune cells. The specific targeting of ovarian cancer cells with low levels of csMUC16 suggests that NK cells are immunoediting the tumor, thereby generating cancer cells that are resistant to immune surveillance.

Molecular Mechanisms for Generation of Immunosuppressive Microenviroment by Cancer Cells

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Cancer cells not only produce various immunosuppressive molecules, but also produce molecules, which induce immunosuppressive cells, including regulatory T cells (Tregs), myeloid-derived suppressor cells, tolerogenic dendritic cell (DC). To make the current immunotherapy more effective, it is important to understand the mechanisms for generation of such a cancer-induced immunosuppressive microenvironment. We have previously reported that culture supernatants from human melanoma cells with enhanced MAPK signaling via mutated BRAF or activated STAT3 signaling, inhibited toll-like receptor-stimulated interleukin (IL)-12 production by human monocyte-derived DC, and treatment of the melanoma cells with lentiviral shRNA specific for BRAF or STAT3 inhibited the suppressive activity of the melanoma cells through inhibited production of IL-10, IL6, and vascular endothelial growth factor. Similarly, suppressive activity of human colon cancer cells with enhanced MAPK signaling via mutated BRAF or RAS, was found to be reduced by treatment with either siRNA for BRAF or MEK inhibitor. Culture supernatants from human melanoma cells with increased β-catenin through β-catenin mutation or antigen-presenting cell defect contained high amount of IL-10, and induced possibly tolerogenic, high IL-10 and low IL-12 producing DC. Treatment of the melanoma cells with siRNA specific for β-catenin resulted in less induction of such DCs accompanied by decreased IL-10 production in melanoma. The melanoma cells transfected with the mutant β-catenin also inhibited interferon-γ production by melanoma-specific cytotoxic T lymphocyte. Human cancer cells transfected with a transcription factor, snail, showed epithelial mesenchymal transition (EMT)-like features, including decreased adhesion, increased invasion, and metastatic ability. These snail transfected cancer cells induced more efficiently immunosuppressive, FoxP3+ Treg from human peripheral blood mononuclear cell by co-culture than the mock transfectants. Subcutaneous implantation of B16 murine melanoma cells transfected with snail showing EMT-like features, induced immunosuppression in vivo through induction of Treg and impaired DC, and intratumoral administration of snail siRNA reduced the tumor through induction of antitumor T cells, indicating that EMT may promote tumor metastasis through not only increased invasive ability, but also immunosuppression. These results indicate that the altered genes in cancer cells are involved in the generation of immunosuppressive microenvironment, and that combined use of their specific inhibitors or siRNA may augment the antitumor activity of the current immunotherapy through simultaneous inhibition of multiple immunosuppressive mechanisms.

Central Role of Tumor-associated CD8+ T-Effector/Memory Cells in Restoring Systemic Anti-tumor Immunity

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Sustained delivery of interleukin (IL)-12 and granulocyte macrophage-colony stimulating factor (GM-CSF) to the tumor microenvironment induces a rapid yet transient activation of preexisting CD8+ T-effector/memory cells followed by cytotoxic CD8+ T-effector expansion. To determine whether the secondary CD8+ T effectors originated from tumor-associated T-effector/memory cells or were primed de novo, activation kinetics of draining lymph node CD8+ T cells were analyzed. Quantitative analysis demonstrated that cytokine delivery promoted a rapid 4-fold increase in CD8+ T-cell numbers in the tumor-draining lymph node (TDLN) between days 1 and 3, which contracted thereafter. In contrast, CD8+ T-cell numbers remained unchanged in the tumor between days 0 and 4, followed by a 3-fold increase between days 4 and 7. Phenotypic analysis showed that TDLN CD8+ T cells up-regulated CD69 and CCR5, and down-regulated CD62L between days 2 and 4, consistent with the development of an activated, periphery-homing phenotype. Pulse labeling with BrdU confirmed that proliferation occurred exclusively in the TDLN between days 1 to 5 and more importantly, demonstrated that CD8+ T-cells migrated directly to tumors. Consistent with these observations, day 4 CD8+ T-cells isolated from the TDLN preferentially homed to tumors following adoptive transfer to tumor-bearing recipients and efficiently killed tumor cells but not control targets, establishing tumor specificity. In contrast, CD8+ T-effector cell priming in the TDLN was completely abrogated in the absence of preexisting tumor-associated CD8+ T-effector/memory cells in studies involving: (a) treatment of mice immediately following surgical removal of the primary tumor and (b) treatment of CD8+ T-cell–depleted mice after selective reconstitution of their TDLN with adoptively transferred, TDLN-homing CD8+ T cells. T-effector cell priming did not occur in interferon-γ knockout mice establishing the critical role of this effector molecule in CD8+ Tem-dependent priming

of antitumor T effectors in the TDLN. These data confirm that intratumoral IL-12 + GM-CSF induces de novo priming of tumor-specific CD8 + T-effector cells in the TDLN and establish the critical role of preexisting intratumoral CD8 + T-effector/memory cells in driving this process.

Proportion of Regulatory T Lymphocytes and Myeloid-derived Suppressor Cells in Peripheral Blood of Patients With Uterine Cervical Cancer

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Backgrounds: In spite of treating cancer patients with immunotherapy by using diverse immune cells or cytokines, many cases did not respond due to immune suppression and tolerance. Regulatory T lymphocytes (Tregs) and myeloid-derived suppressor cells (MDSCs) are known for immune suppressive cells. Tregs are defined as a T lymphocytes population that functionally suppresses the immune response and expresses CD4 and CD25 on the surface of cells and Forkhead box P3 as a transcription factor in cytosol. MDSCs, expressing CD11b and CD33 on the surface of cells, are expanded in patients whose T-cell function does not work efficiently. It was reported that the depletion of the MDSCs restored interferon-γ production and T-cell proliferation. Many studies have indicated that Tregs and MDSCs play a major role in disturbing the effect of immunotherapy. However, it is not clear which conditions occur to the immune suppressive cells in patients with uterine cervical cancer. To improve immunotherapy, we need to find out the features of immune system against tumor. In this study, therefore, we investigated the proportion of Tregs and MDSCs in patients with uterine cervical cancer.

Materials and Methods: Peripheral blood was sampled from 25 patients with uterine cervical cancer who were treated in Asan medical center from July 2007 to June 2008 and from 28 healthy women as controls. We obtained the peripheral blood mononuclear cells by the Ficoll-Hypaque gradient density centrifuge method and analyzed the phenotype with flow cytometry by using anti-CD4, anti-CD25, and FoxP3 antibodies for Tregs and anti-CD11b and anti-CD33 antibodies for MDSCs.

Results: The immune suppressive cells were highly found in the patient group compared with the healthy group. The proportion of CD4 + CD25 + FoxP3 + Tregs significantly increased in the patient group as 9.99% ± 3.26% whereas the healthy group indicated 6.27% ± 3.54%, ($P < 0.05$). The proportion of CD4 + CD25high + FoxP3 + Tregs was slightly higher in the patient group (1.92% ± 1.17%) than in the healthy group (1.38% ± 0.88%), however, it was not significant ($P = 0.152$). In case of MDSCs, the proportion of CD11b + CD33 + MDSCs was significantly increased in the patient group as 23.34% ± 15.78% whereas the healthy group indicated 15.78% ± 8.61% ($P < 0.05$).

Conclusions: This study shows that the immune suppressive cells, such as Tregs and MDSCs, increase in patients with uterine cervical cancer. Therefore, controlling the expression of Tregs and MDSCs could make the immunotherapy more effective for patients with cervical cancer who have a negative response to the conventional immunotherapy.

Immunoglobulin-like Transcript 3 (ILT3) is Expressed by Myeloid Derived Suppressor Cells in the Tumor Microenvironment of Melanoma Patients

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Immunoglobulin-like transcript (ILT) 3 is an inhibitory receptor expressed on antigen-presenting cells that block cell-mediated immune responses through de-differentiation of effector T cells and induction of regulatory CD4 + and CD8 + T cells. The level of ILT3 expression is associated with graft tolerance in cardiac transplantation patients and an increase in regulatory T cells in myocardial tissue. We have previously demonstrated an increase in soluble ILT3 in the serum of patients with metastatic melanoma although the source of soluble ILT3 has not been clearly defined. Immunostaining of the tumor microenvironment of metastatic melanoma patients suggested that ILT3 was expressed by CD68 + monocytic cells surrounding established tumor tissue. To better characterize these cells we sought to better define the myeloid-derived suppressor cell (MDSC) population in patients with melanoma. Peripheral blood mononuclear cells were collected from 10 normal donors and 13 melanoma patients. We detected 4 distinct subpopulations of myeloid cells within the peripheral blood mononuclear cell compartment: CD14hi + CD11b +, CD14 + loCD11b +, CD33hi + CD13 +, and CD33 + loCD11b +. These populations could be further separated based on HLA-DR expression to obtain CD33hi + CD13 + HLA-DRdim/ − and CD14hi + CD11b + HLA-DRdim/ − cells using magnetic beads. Functional assay showed that only HLA-DRdim/lo myeloid cells exhibited suppressor activity as defined by T-cell inhibition using standard MLR proliferation assays. The frequency of MDSC ($CD14 + CD11b + HLA - DR -$) was significantly increased in melanoma patients compared to healthy donors ($P = 0.0058$). ILT3 expression was also significantly increased in MDSC isolated from melanoma patients compared to MDSC derived from healthy donors ($P = 0.0366$). Further analysis of the tumor microenvironment in metastatic melanoma patients by flow cytometry suggested that ILT3 + hiMDSC accumulated to an even greater extent than in peripheral blood, although there was considerable inter-patient variability. Together, these data imply that ILT3 + hiMDSC may be a source of ILT3 in patients with established melanoma. These cells could block differentiation of effector T cells and promote the expansion of regulatory T cells within the tumor microenvironment. Further studies aimed at blocking ILT3 expression and/or binding are planned to determine if this will significantly improve local effector T-cell responses and the success of tumor immunotherapy.

Hemoglobin-beta as a Tumor-rejection Antigen Allowing Immune Targeting of the Tumor-associated Stroma

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Material and Methods: BALB/c bone marrow-derived dendritic cells (DCs) were infected with control (Ad.ψ5) or interleukin (IL)-12 encoding (Ad.IL12) adenoviruses for 48 h at 37°C, then injected into established day 7 subcutaneously (H-2d) CMS4 tumors, with repeat injections provided on day 14. Tumor growth was monitored through day 28. Specific T-cell responses at day 28 were assessed against tumor-derived peptides using an interferon-γ enzyme-linked immunosorbent assay and bioactive peptides sequenced using mass spectrometry. The relevance of sequenced peptides was determined by vaccination of mice in advance of challenge with a range of syngeneic tumor cell lines including sarcomas (CMS4, MethA), a breast carcinoma (4T1), or a colon carcinoma (CT26). Expression of the relevant target antigen in tumor cells in vitro and primary lesions in vivo was analyzed using reverse transcription-polymerase chain reaction and immunofluorescence microscopy, respectively.

Results: Intratumoral injection of DC infected with Ad.IL12 (DC.IL12), but not the control vector, resulted in the rejection of CMS4 tumors, which correlated directly with the magnitude of the splenic anti-CMS4 CD8 + T-cell response. Protective CD8 + T cells recognized a limited series of naturally processed and H-2Ld-presented peptides, at least 2 of which derive from murine hemoglobin-beta (HBB). When naive mice were vaccinated with DC.IL12 pulsed with HBB peptide as a vaccine, they were subsequently protected against challenge with CMS4, MethA, 4T1, or CT26 tumor cells, all of which failed to express HBB (reverse transcription-polymerase chain reaction). Consequent IFM analyses revealed that the major cell types expressing HBB within the tumor lesion were NG2 + pericytes that were found in lower frequencies in tumor biopsies harvested from HBB peptide-vaccinated animals. Importantly, no deleterious effects were observed against red blood cells or erythroid precursors in these same vaccinated mice.

Conclusions: In this model, HBB expressed by tumor-associated stromal target cells (pericytes) appears to represent a therapeutically relevant target for protective CD8 + T cells. Anti-HBB T cells protect against all tested solid (vascularized) tumor cell types evaluated and do not appear to be associated with pathologic autoimmunity.

Tumor Treg Potently Abrogate In Vivo Antitumor T Cell Priming

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CD4+CD25+ but not CD4+CD25− T cells taken from tumor-bearing mice highly expressed Foxp3 and exhibited potent suppressive activity in vitro and in vivo. The suppressive capacity of Foxp3+CD4+CD25+ regulatory T cells (Treg), which were elevated in tumor-bearing mice (tumor Treg), was comparable to Treg from naive mice (naive Treg) in vitro. Neutralizing transforming growth factor-β or cytotoxic T lymphocyte antigen-4 signaling attenuated the ability of tumor Treg in inhibition of naive CD8+ T-cell activation in vitro. Interestingly, tumor Treg potently dampened dendritic cell capacity in vitro when compared with naive Treg. In vivo neither tumor nor naive Treg suppressed adoptively transferred tumor-primed CD4+ T-cell–induced antitumor immunity at effector phase, consistent with their inability to in vitro inhibit tumor-specific lymphocyte effectors generated by adoptively transferred tumor-primed CD4+ T cells. However, in vivo tumor Treg but not naive Treg abolished tumor-specific CD8+ T-cell priming. It bears the in vivo observation that tumor Treg but not naive Treg abrogated protective antitumor activity induced by adoptively transferred tumor-primed CD4+ T cells at priming phase. Taken together, the data indicates that tumor Treg in vivo potently abrogate tumor-specific CD8+ T-cell priming, thereby inhibiting adoptively transferred tumor-primed CD4+ T-cell–induced antitumor activity. Therefore, the finding provides important insights for the understanding of tumor Treg-mediated suppression and implications for the development of rational strategies to in vivo manipulate Treg.

Expressing T_H1 or T_H2 Cytokines in the Bladder and Their Effects on Tumor Growth in a Murine Orthotopic Bladder Tumor Model

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Single immune related genes have been used to successfully induce tumor regression in murine models of bladder cancer. To gain insight into the cause of this amazing ability of diverse genes we previously studied tumor-induced changes in gene expression in the bladder using an orthotopic model of bladder cancer. Based on our results, the presence of tumors cells induced both protumor and antitumor gene expression in the bladder. We hypothesized that a slight imbalance of the genes expressed in the bladder with an increase in those favoring antitumor activities may be sufficient to eradicate tumors explaining the diversity of favorable gene responses noted in the literature. To confirm our hypothesis we studied the effect of expressing T_H1 and T_H2 genes in the bladder.

Mice (n = 6/group) were treated with Poly-L-Lysine and then implanted with the murine bladder cancer cell line MB49-secreting PSA (MB49-PSA) and the mice were treated to nonviral gene therapy twice a week after day 5. Cytokine genes (IL-10, GM-CSF) were cloned into pBudCE4.1 (Invitrogen). The therapy groups consisted mice treated with intravesical instillations of pBudCE4.1 control vector alone or pBud-cytokine gene. Each mouse received 0.25 μg of plasmid DNA [transfection mixture: 2.5 μg DNA + 20 μg DOTAP (Roche) + 40 μg methyl-β-cyclodextrin solubilized cholesterol, MBC (Sigma) made up to 1 mL RPMI]. Mice were terminated 2 days after the fourth instillation. Control tumor bearing mice did not receive any therapy. mRNA expressions in the bladder were analyzed using a TaqMan® Low Density Mouse Immune Panel (Applied Biosystems) containing a panel of 93 immune-related genes and endogenous controls. Gene expression was confirmed using real-time polymerase chain reaction analysis of the mRNA from the other murine samples.

At a glance the profile of gene expression changes between the different cytokine treated bladders were dramatically different depending on whether the cytokines were T_H1 or T_H2 cytokines. The cytokine gene used for transfection was always well expressed in the bladder tissue confirming the success of transfection. The plasmid vector itself induced some changes in gene expression. Compared to untreated tumor bearing bladders IL-10 increased (C3, Ccl19) and decreased (FasL, Cd8a, Cd40L, Gzmb1, Cd38, Socs2, Ptprc, Ccl3, bcl2L1, H2-Eb1) the expression of these genes more than the vector alone. Most of these genes were involved in the anti-tumor response. GM-CSF increased the expression of monocyte and macrophage attracting genes.

Tipping the balance between pro and anti-tumor genes affects tumor growth in the bladder. Attempts to hit multiple gene targets may enhance the response to therapy.

Soluble VEGF Receptor Production From GM-CSF-Stimulated Human Monocytes is Enhanced Under Hypoxic Conditions

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Accumulation of macrophages within a tumor is associated with poor clinical outcome. It is thought that macrophages contribute to tumor progression through their secretion of the pro-angiogenic factor vascular endothelial growth factor (VEGF). We have previously observed that monocytes treated in vitro with the cytokine GM-CSF produce a soluble form of the VEGF receptor (sVEGFR-1), which sequesters VEGF from biological activity. Furthermore, intratumoral administration of GM-CSF reduced angiogenesis and inhibited tumor growth in a murine model of breast cancer, an effect that was dependent on production of sVEGFR-1 by tumor-associated macrophages. The VEGF and the VEGFR-1 promoters both contain a hypoxia regulatory element (HRE), which binds the HIF transcription factors under hypoxic conditions. Based on this observation, we examined VEGF and sVEGFR-1 production from monocytes cultured at various O_2 levels (ambient O_2, 5% O_2, or 0.5% O_2). At all O_2 levels examined, GM-CSF–stimulated monocytes secreted > 10-fold the amount of sVEGFR-1 as compared to unstimulated monocytes at the same O_2 level. In addition, the amount of sVEGFR-1 production observed from both unstimulated and GM-CSF–stimulated monocytes increased with decreasing levels of O_2. In order to confirm that the sVEGFR-1 was capable of sequestering VEGF, supernatants were assessed using an ELISA that detects free (bioavailable) VEGF, but does not detect VEGF that is bound to sVEGFR-1. VEGF detection was significantly inhibited in the supernatants of monocytes that had been stimulated with GM-CSF. Increased sVEGFR-1 production at low O_2 was observed with the lowest concentration of GM-CSF tested (0.1 ng/mL), and peaked at 10 ng/mL. Stimulation of monocytes with GM-CSF in the presence of $CoCl_2$, which prevents the O_2-medated degradation of the HIFs, also elevated sVEGFR-1 production and inhibited VEGF detection. In order to determine which of the HIFs were responsible for the sVEGFR-1 production, we utilized mice with a genetic deletion of HIF-1α in the monocyte/macrophage lineage. HIF-1α knockout macrophages cultured with GM-CSF at hypoxia secreted reduced amounts of VEGF as compared to WT littermates, while sVEGFR-1 secretion was unaffected. In contrast, siRNA-mediated knockdown of HIF-2α inhibited the production of sVEGFR-1 in response to GM-CSF and low O_2. Although hypoxia is generally thought to promote angiogenesis, these results indicate that angiogenesis can be inhibited by hypoxia in a GM-CSF–rich environment. Furthermore, these results suggest that local administration of GM-CSF could be an effective means of limiting angiogenesis in patients with cancer.

L-Arginine Availability Regulates Cyclin D3 mRNA Stability in Human T Cells by Controlling HUR Expression

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Depletion of extra cellular levels of L-arginine (L-Arg) by arginase I-producing MDSC inhibit CD3z expression and blocked T cell proliferation, which may impair the potential therapeutic benefit of immunotherapy. L-Arg starvation impairs T cell proliferation by arresting cells in G0 to G1 phase of the cell cycle, which is associated with an inability to up-regulate cyclin D3. The regulation of cyclin D3 by L-Arg starvation included a low rate of transcription, a decreased mRNA stability and an impaired translation. We aimed to determine the posttranscriptional mechanisms leading to a decreased stability of cyclin

D3 mRNA in T cells cultured under L-Arg starvation. We found that 3'UTR within the cyclin D3 mRNA contains response elements, which inhibit mRNA stability in the absence of L-Arg. The increased cyclin D3 mRNA stability observed in T cells cultured in the presence of L-Arg was associated with a higher cytoplasmic expression of RNA-binding protein (RBP) HuR. Furthermore, HuR binds cyclin D3 mRNA in vitro and endogenously in T cells cultured in the presence of L-Arg, but not in T cells cultured in the absence of L-Arg. Silencing HuR expression in primary T cells using siRNA leads to a decreased cyclin D3 mRNA stability and a lower expression of cyclin D3 protein. We have previously shown that T cells from GCN2 knockout mice proliferate in the absence of L-Arg. As expected, T cells from GCN2 knockout mice, but not from wild type mice, cultured in the absence of L-Arg up-regulated HuR and did not show a decreased cyclin D3 mRNA stability. These results therefore suggest that in T cells cultured in the absence of L-Arg, GCN2 impairs cyclin D3 mRNA stability by blocking the expression of HuR. These data contribute to understand a central mechanism by which cancer and other diseases characterized by high arginase I production may cause T-cell dysfunction.

Epithelial-Mesenchymal Transition in Renal Cell Carcinoma (RCC): Influence on Antigen Expression and Immune Recognition

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Epithelial-mesenchymal transition (EMT) is a central mechanism that governs embryonic development including mesoderm formation and neural tube formation. Recently, it has been discovered that EMT can also be part of tumor progression characterized by loss of cell adhesion and increased cell mobility. Hereby tumors show a diversity of phenotypes and malignant potential. The process of EMT in tumor progression allows quiescent epithelial cells to acquire a mesenchymal-like phenotype, which enables them to escape from the solid tumor, override the basement membrane and intravasate into blood or lymphatic vessels where they are passively transported to distant organs. At secondary sites, solitary carcinoma cells can extravasate from the vessels and form new carcinomas (metastasis) through the reverse process, mesenchymal-epithelial transition.

Several oncogenic pathways and growth factors have been shown to induce or potentiate EMT. The hallmark of EMT is loss of E-cadherin caretaker of the epithelial state–which is mediated, among other mechanisms, by transcriptional repressors or epigenetic changes. Many cell lines show partial or complete EMT in vitro. However, EMT in renal cell carcinoma (RCC) has so far not been described in situ.

The goal of this project was to analyze the status of EMT in RCC in vitro and in situ, to determine how the epithelial or mesenchymal phenotype can be modulated in vitro and if this impacts on recognition by immune effector cells.

In situ studies of tumors showed loss of E-cadherin expression especially in sarcomatoid areas of RCC. Furthermore, we found no differential expression of E-cadherin repressors in E-cadherin positive versus E-cadherin negative tumors, suggesting that other mechanisms are responsible for E-cadherin regulation in RCC.

In vitro, E-cadherin expression could be further down-regulated by growth factors known to induce EMT, such as transforming growth factor-β or fibroblast growth factor-2, and up-regulated at the transcript level by epigenetic modulations, like promoter demethylation, or at the protein level by transfection of in vitro transcribed mRNA encoding E-cadherin. Following transfection of E-cadherin in vitro transcribed mRNA, innate effector cells were shown to be inhibited by E-cadherin expression and loss of E-cadherin may favor killing of tumor cells by innate effector cells undergoing EMT.

Indoleamine 2,3-dioxygenase Expression in Renal Cell Carcinoma

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Indoleamine 2,3-dioxygenase (IDO) catalyzes the rate-limiting step in tryptophan metabolism. It is expressed constitutively in many normal tissues and can also be induced by various stimuli. IDO has been found to be overexpressed in several types of cancer and is thought to contribute to tumor-mediated immunosuppression by reducing free tryptophan and increasing tryptophan metabolites in the tumor microenvironment. IDO also drives differentiation of regulatory T cells, which in turn trigger increased IDO expression in a positive feedback loop. We hypothesized that IDO is increased in renal cell carcinoma (RCC) and measured its expression by immunohistochemistry. Our preliminary data have shown that IDO is expressed in the normal adjacent kidney (NAK), but not the tumor itself, in a subset of RCC patients. Higher IDO expression in NAK correlated with shorter relapse periods. We also analyzed the urine samples of these patients using reverse-phase high performance liquid chromatography and found increased levels of tryptophan metabolites, including quinolinic acid. These results provide evidence for the role of IDO in promoting immunologic tolerance in the tumor microenvironment. In ongoing studies we are using immunohistochemistry to measure expression of IDO, quinolinic acid, and the Treg transcription factor, Foxp3, in RCC tumors and NAK.

Molecular Regulation of MDSCs by COX-2 and TK Inhibitors in a Transgenic Murine Mammary Cancer Model

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Myeloid-derived suppressor cells (MDSCs) are potent immune regulatory cells associated with tumor progression and growth. Expansion and activation of MDSCs can result in tumor evasion from host immunity and down-regulation of T-cell number and function. Our current hypothesis, is that the strategic down-regulation of MDSCs is critical to the success of immune and vaccine based adjuvant interventions. We report an indirect relationship between MDSCs and T cells and a direct relationship between MDSCs and tumor initiation, progression, growth and metastasis in NeuT transgenic mice on an FBV background that spontaneously develop mammary carcinomas. Quantitative reverse transcription-polymerase chain reaction analysis of growth factors, cytokines, and enzymes with immune regulatory properties have suggested molecular mechanisms for the expansion, activation and mechanism of action by MDSCs in this animal model of mammary cancer. Further, studies targeting manipulation of the molecular effectors; cyclooxygenase-2 (COX-2) and tyrosine kinase (TK) inhibitors have resulted in the regulation of MDSC number and function, as well as, tumor induction, progression, growth, and metastasis.

In these studies, more than 90% of the transgenic mice developed mammary tumors; with the development of the first and second tumor occurring at a median of 265 and 329 days, respectively, and a median survival time of 432 days. In FBV-neuT transgenic mice, MDSCs increased in the spleen in association with tumor progression. In addition, MDSCs and Tregs were observed as tumor infiltrating leukocytes (TILs). These cells were isolated by density gradient separation of non-parenchymal cells following collagenase-DNase digestion. The TILs expressed higher levels of ARG1, NOS2, VEGFA, and lower levels of interferon-γ and TCR-zeta as compared to tumor bearing spleen cells. Interestingly, MDSCs in the TILs correlated with tumor volumes and expression of GM-CSF and NOS2. The tumor volumes also correlated with the total number of CD4 cells in the spleen. Further, molecular intervention using the COX-2 inhibitor; celecoxib at 80 mg/kg in drinking water; or the TK inhibitor; sunitinib at 40 mg/kg/d, po delayed tumor induction, and reduced the incidence and growth of tumors. In association with these improved clinical outcomes, MDSCs expansion was reduced and T cells numbers increased. Treatment with sunitinib significantly reduced stem cell factor and VEGF transcript levels that are increased in association with tumor growth. We conclude that both COX-2 and TK inhibitors have therapeutic activity, in association with a down regulation of MDSCs, reduced T-cell lymphopenia and partial normalization of the inflammatory cytokine storm induced by tumor progression.

Effect of Arginase II on L-Arginine Depletion and Cell Growth in Murine Cell Lines of Renal Cell Carcinoma

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Background: L-arginine is the common substrate for 2 isoforms of arginase. Arginase I, highly expressed in the liver and arginase II mainly expressed in the kidney. Arginase I-producing myeloid derived suppressor cells have been shown to inhibit T-cell function by the depletion of L-arginine in lung and pancreatic cancer. Arginase II on the other hand has been shown to be increased in breast, colon and prostate cancer and is thought to metabolize L-arginine to L-ornithine needed to sustain rapid tumor growth. Thus, L-arginine metabolism may play a dual role in both tumor growth and in the induction of T-cell dysfunction. These factors compromise the microenvironment that may help tumors to evade the immune response. The role of arginase II in renal cell carcinoma (RCC) and its effect on the depletion of L-arginine and cell growth have not been investigated.

Methods and Purpose: In the present study, using murine renal carcinoma cell lines CL-19, CL-2 and Renca, we investigated the activity, expression and the effect of arginase II on tumor cell proliferation and L-arginine depletion and its effect on the expression of CD3ζ. The arginase inhibitor nor-NOHA was used to substantiate the effect of arginase II on cell growth and L-arginine depletion. Amino acid levels were tested by HPLC.

Results: Our results show that murine RCC cell lines express only arginase II and were able to deplete L-arginine from the medium after 48 hours in culture. Cell growth was independent of the amount of arginase activity expressed by the cells as seen by the significant higher proliferation of Renca cells, which express low arginase activity. nor-NOHA significantly ($P = 0.01$) reduced arginase II activity and suppressed cell growth in cells exhibiting high arginase activity. The depletion of L-arginine also induced a decrease in the expression of CD3ζ a key element for T-cell function.

Conclusions: The results of this study show for the first time that indeed arginase II produced by RCC cell lines depletes L-arginine resulting in decreased expression of CD3ζ. These results indicate that RCC cell lines depend on L-arginine for growth. The intracellular arginase activity may be the major factor determining the requirement for L-arginine. In addition, these cells can also use other amino acids (ie, L-glutamine) to supply the deficiency of L-arginine necessary for tumor growth. Blocking arginase may lead to a decrease in RCC cell growth and aid in restoring immune function by increasing L-arginine availability for T-cell use. Understanding the interplay between arginase II biology and its interaction with the immune system may provide future therapeutic benefits to treat patients with RCC.

Persistent High Grade Cervical Dysplasia Excludes CD8+ T Cells

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Persistent mucosal infection with human papillomavirus (HPV) is the cause of virtually all squamous cervical cancer (SCC). High-grade cervical dysplasia (CIN3), the lesion, which is the immediate precursor to SCC, is associated with integration of the HPV genome into the host genome, and subsequent constitutive expression of the HPV E6 and E7 oncoproteins. Because both E6 and E7 are functionally required for disease, they present compelling targets for immunotherapeutic strategies. We have established a prospective cohort of subjects with CIN3, who are followed conservatively for a brief 15-week observational protocol prior to undergoing standard therapeutic resection. In this window, no subject has had progression of disease. Neither have we identified occult, unsuspected invasive disease in any subject at the time of resection at week 15 (Tweek15). In fact, up to 25% of high grade lesions associated with HPV16 undergo complete regression in this window, which is presumably immunologically mediated. We measured CD8 T-cell responses to HPV16 antigens in peripheral blood specimens obtained longitudinally from study participants, and were unable to identify responses that correlated with disease outcome. Therefore we examined the cervical compartment to determine the extent to which lesions contained immune cells.

In normal cervical mucosa, CD8+ cell infiltrates were detectable in low numbers, predominantly distributed along the superficial lamina propria immediately subtending the epithelial basement membrane. Immune cells isolated from normal cervix were overwhelmingly comprised of antigen-experienced T cells, which expressed epithelial addressins CLA and CCR4. Compared to the peripheral blood compartment, very few B cells or NK cells were detected. Compared to normal cervical mucosa, CIN3 lesions were associated with a higher intensity of CD8+ infiltrates ($P < 0.0001$), which were greater in the lamina propria compared to the epithelial compartment ($P < 0.0001$). Both the higher density and the localization of CD8+ cells in the lesional tissue compared to immediately adjacent normal mucosa suggest recruitment of this population. Moreover, in lesions, which were still present (persistent CIN3) at the time of resection (Tweek15), we observed higher intensity of CD8+ infiltrates localized to the lesion site compared to baseline (T0). In contrast to the lamina propria infiltrates, in persistent CIN3 lesions, infiltration of the epithelial compartment with CD8 cells did not change appreciably. This constellation of findings suggests that persistent CIN3, despite expression of potentially immunogenic viral proteins, excludes CD8 T cells.

Increasing Immunostimulatory Ability of Tolerogenic APCs Enhances Anti-tumor Immunity

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One obstacle in adoptive immunotherapy of cancer is the loss of effector function by tumor-specific CD8+ T cells. Our laboratory previously demonstrated that following adoptive transfer into prostate tumor-bearing mice, CD8+ tumor-specific T cells become activated in the periphery and traffic to the tumor. However, upon infiltration into the prostate tumor microenvironment, the cells were observed to be functionally tolerant of their cognate antigen. Because the potency of tumor-specific T cells is regulated by many factors, including tumor-associated tolerogenic antigen-presenting cells (APCs), in the current study, we examined the function and phenotype of the APCs present in both the prostate tumor microenvironment as well as the tumor draining lymph node. Using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, we have observed that the largest population of APCs within the prostate tumor microenvironment were CD11cint/B220+/mPDCA-1+, which are reportedly characteristic of APCs that are poor presenters of Ag. Furthermore, we noted that these APCs produced elevated levels of molecules that are known to suppress T cell responses including indoleamine 2, 3 dioxygenase (IDO) and arginase I (ARG 1), as well as ligands such as PDL-1 and FASL, which can induce anergy, exhaustion, and programmed cell death in T cells that express the PD-1 and FAS receptors. Interestingly, we demonstrate that by inhibiting the activity of the tolerogenic enzymes IDO and ARG 1, or by blocking receptor ligation of PD-1, tolerance induction of tumor specific T cells was delayed in vivo. Further studies revealed that providing tumor-specific CD4+ T cell help enhanced APC expression of co-stimulatory molecules and increased their ability to stimulate proliferation of naive CD8+ T cells in vitro. Our data demonstrate that the tolerization of tumor-infiltrating CD8+ T cells may be dependent upon the phenotype, activation, and function of the APCs within the tumor microenvironment. These findings have critical importance for the design of novel immunotherapies that sustain T-cell responses to tumor antigens to elicit more potent, long-lasting tumor immunity.

Evidence for Selection of a Resistant Tumor Microenvironment Following Successful Clinical Response to a Multi-peptide + IL-12 Melanoma Vaccine

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We recently have identified a gene expression signature in melanoma metastases that correlates with clinical response to a melanoma vaccine utilizing 4 tumor antigen peptides and IL-12. When feasible, patients in this trial are being followed longitudinally to monitor the evolution of the T-cell response and changes in tumor biology. Here we describe the features a patient who initially responded then recurred 3 years after vaccination. In 2004, a 51-year-old male diagnosed with melanoma was recruited to participate in this vaccine trial. He was immunized subcutaneously with irradiated (2000 rad) autologous PBMCs pulsed with Melan-A, gp-100, NA-17, and MAGE-3 peptides along with rhIL-12 every 3 weeks for 6 months. A pre-treatment tumor biopsy revealed a tumor microenvironment that was "favorable", containing transcripts for T cell-recruiting chemokines. Following the third immunization, robust T-cell responses were observed against all 4 peptides. Clinically he experienced a durable partial response. He was then monitored by routine follow-up until 2007 when a clinical recurrence was detected in the form of a new pelvic mass. Analysis of the T-cell response in the peripheral blood at that time revealed persistent reactivity against Melan-A and NA-17. Biopsy and gene expression profiling of the recurrent tumor, however, revealed a significant down-regulation of transcripts encoding key chemokines, as well as up-regulation of transcripts linked to more aggressive tumor biology. Immunohistochemistry revealed exclusion of CD8+ T cells from the center of the tumor mass. Our results suggest that metastatic melanoma may have the potential to become selected under immune pressure to develop a tumor microenvironment that is resistant to the effector phase of the antitumor T-cell response.

Blockade of PD-1/PD-L1 Interactions is Paradoxically Detrimental in a T Cell Adoptive Transfer Tumor Therapy Model

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Immunoregulatory mechanisms active within the tumor microenvironment can dominate even successfully primed antitumor immune responses, leading to tumor outgrowth. We have developed a murine adoptive transfer model aimed at reversing 2 putative negative regulatory mechanisms-tumor-induced T-cell anergy and extrinsic suppression by regulatory T cells (Tregs). We found that T-cell anergy could be prevented upon homeostatic proliferation (HP) of bulk splenic T cells from C57BL/6 mice into lymphopenic hosts. Furthermore, Tregs could be depleted prior to adoptive T-cell transfer using anti-CD25 microbeads. The combination of HP and $CD25^+$ T-cell depletion led to potent rejection of B16.SIY tumors (B16 cells transduced to express the SIYRYYGL antigen) in the prophylactic and 3-day preimplanted tumor settings. When B16.SIY tumors were established 8 to 10 days prior to transfer of CD25-depleted T cells, they were partially controlled but not rejected. Vigorous SIY-specific T-cell responses were detected in the spleen, suggesting the possibility that additional negative regulatory mechanisms were active at the effector phase of the immune response. To determine if the PD-1/PD-L1 pathway was negatively regulating anti-tumor immune responses, $RAG2^{-/-}$ mice were challenged with B16.SIY, followed 8 to 10 days later by adoptive transfer of CD25-depleted T cells from wildtype or PD-$1^{-/-}$ mice. PD-$1^{-/-}$ T cells did not augment rejection of established B16.SIY tumors. Surprisingly, when SIY-specific T-cell responses were analyzed with SIY/K^b tetramers and IFN-γ ELISPOT, there was a paradoxical decrease in both expansion and effector function of adoptively transferred PD-$1^{-/-}$ T cells. Blunted SIY-specific T-cell response were observed at all time points tested, arguing against altered kinetics of the immune response. Similar results were seen when using PD-L1 blocking mAb, arguing against a defect due to an altered T-cell repertoire in PD-1^- mice. The difference in T-cell priming was not due different rates of HP, as wildtype and PD-$1^{-/-}$ T cells proliferated at a similar rate in lymphopenic tumor-bearing recipients. In contrast to the adoptive transfer model, when wildtype or PD-$1^{-/-}$ mice were directly challenged with B16.SIY cells, endogenous immune responses were augmented in PD-$1^{-/-}$ mice as expected. Together, these data suggest either that the inhibitory function of PD-L1/PD-1 interactions depends on the presence of Tregs, is reversed by homeostatic proliferation, or is otherwise altered by the process of adoptive T-cell transfer. Care should be taken when applying PD-L1/PD-1 blockade in specific immunotherapeutic settings.

TUMOR TARGETING MONOCLONAL ANTIBODIES

Targeting Human B Cell Chronic Lymphocytic Leukemia With a Monoclonal Antibody Specific for the Receptor Tyrosine Kinase ROR1

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Rituximab, a mouse-human chimeric monoclonal antibody (mAb) is being widely used as an immunotherapeutic agent for the treatment of human B cell malignancies including B cell chronic lymphocytic leukemia (B-CLL). The target antigen, CD20, is expressed not only by the malignant B cells but also by normal B cells. Gene expression profiling on a genomic scale led to the discovery of a distinctive B-CLL gene signature composed of genes selectively expressed by B-CLL cells. However, the expression and function of the corresponding proteins and their suitability as targets for therapeutic intervention remains largely unexplored. Using polyclonal antibodies, we have recently reported the selective expression of one such protein, the receptor tyrosine kinase-like orphan receptor 1 (ROR1), on the surface of B-CLL cells. By contrast, nonmalignant blood cells in B-CLL patients, as well as healthy donors including normal B cells and normal adult tissues/organs tested do not express ROR1 protein on the cell surface. Such a restricted, uniform, and constitutive cell surface expression of ROR1 protein in B-CLL provides a strong incentive for the development of targeted therapeutics such as monoclonal antibodies. We immunized mice with a fusion protein consisting of the human Fc domain and the extracellular domain of human ROR1, and generated hybridomas secreting mAb against human ROR1. The 4 mAbs were characterized by ELISA, flow cytometry, and surface plasmon resonance measurements. All 4 mAbs are of IgG1-kappa isotype and showed specific binding and recognition of purified human ROR1. Both the hybridoma supernatants and affinity purified IgG of these mAbs demonstrated specific recognition of cell surface ROR1 on ROR1-expressing transfectants, and more importantly, on primary B-CLL cells from all patients tested. The antigen binding avidity of one of these mAb (2A2) was in the range of 100 pM with a very low dissociation rate. Based on these results we suggest that our anti-ROR1 mAb can serve as a starting point for the development of a therapeutic agent for human B-CLL. Construction of a mouse-human chimeric mAb would facilitate additional effector functions as well as minimize its immunogenicity in patients.

Antibody and Small Modular Immune Pharmaceutical Therapies for Patients With Chronic Lymphocytic Leukemia: A Major Step Forward

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Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia and is currently not curable with available therapy. For several decades different cytotoxic therapies have been introduced with only modest improvement in observed response and time of treatment remission. The introduction of the 2 therapeutic monoclonal antibodies rituximab and alemtuzumab has greatly impacted the therapy of CLL. Rituximab when combined with fludarabine or fludarabine and cyclophosphamide increases the complete remission rate significantly, promotes remissions lasting greater than 5 years in a subset of patients and may prolong survival. Similar promising results have been observed with alemtuzumab when applied as a consolidation therapy for eliminating minimal residual disease. Given the success of therapeutic antibodies in CLL, we have taken an active role in exploring several new therapeutic antibodies in the laboratory and clinic. Our laboratory interest has recently transitioned to studying a different class of drugs, small modular immune pharmaceuticals (SMIP). Several SMIP agents have been constructed using a single chain variable region (scFv) linked to a modified human IgG1 hinge, C_H2 and C_H3 domains. CD37 SMIP is one such agent that targets CD37, a lineage-specific B-cell antigen that represents an attractive target for immunotherapy in B-cell malignancies. We have demonstrated that CD37 SMIP promotes significant induction of apoptosis and antibody dependent cellular cytotoxicity (ADCC) but not complement mediated

cytotoxicity by CD37-SMIP against B-cell lymphoma/leukemia cell lines and primary CLL cells. The apoptosis induced by CD37-SMIP was correlated with levels of CD37 surface expression and occurred independent of caspase activation. Most notably, CD37 SMP mediates apoptosis and ADCC significantly better than alternative antibodies used for CLL including alemtuzumab and rituximab. We have examined which effector cells are responsible for ADCC and have identified that natural killer (NK) cells but not naïve or activated monocytes mediate CD37-SMIP dependent ADCC function in vitro. Interestingly, CD37-SMIP conferred significant protection from disease progression in vivo in a Raji cell xenograft SCID mouse model of disseminated leukemia/lymphoma with a dramatic improvement in survival following treatment. Depletion of NK cells in mice resulted in diminished efficacy of CD37-SMIP further supporting the in vivo importance of NK cells in SMIP-mediated therapeutic efficacy. Overall our data suggest that the CD37-SMIP is a promising therapeutic agent against CD37+ B-cell malignancies that warrants further clinical development. This talk will focus on new antibody and SMIP based therapies coming forward for the treatment of CLL and related lymphoproliferative disorders.

Cetuximab Mediated Antibody Dependent Cellular Cytotoxicity (ADCC) by NK Cells Expressing Polymorphic FC Gamma Receptor (FCγR)IIIA

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Despite cetuximab's clinical efficacy against squamous cell carcinoma of the head and neck (SCCHN) only 10% to 20% of the patients receiving it demonstrate responses. We previously demonstrated that the epidermal growth factor receptor (EGFR)-specific monoclonal antibody cetuximab can mediate antibody-dependent cell cytotoxicity (ADCC) against SCCHN cells, but factors responsible for variability between donors, such as polymorphisms in Fcγ receptor (FcγR) and level of EGFR expression have not been determined. ADCC assays were performed using cetuximab treated SCCHN cell lines expressing different levels of EGFR and enriched natural killer (NK) cells or unfractionated peripheral blood mononuclear cell (PBMC0 from SCCHN patients or healthy donors. Effector cells were characterized for their FcγRIIIa-158 genotype and analyzed by flow cytometry for CD69 and CD107a expression. Supernatants from these assays were analyzed using multiplexed enzyme-linked immunosorbent assay for their cytokine and chemokine secretion. NK cells and SCCHN patients' PBMC with poor ADCC responses were treated with interleukin (IL)-2 or IL-15 and used in ADCC assays. Cetuximab-mediated ADCC against SCCHN cell lines varied with the NK cell FcγRIIIa-158 polymorphisms (VV > VF > FF, $P < 0.001$) and was dependent of antibody concentration and level of EGFR expression. Furthermore, these polymorphisms correlated with CD69 and CD107a expression by effector cells and their secretion of IFN-γ, tumor necrosis factor-α, IL-8, MIP-1α, and MIP-1β. Treatment of effector PBMCs with IL-2 or IL-15 enhanced ADCC activity in both poor responder PBMCs, leading to increased effector cell activation phenotype and cytokine secretion genotype.

The importance of FcγRIIIa polymorphisms in cytotoxicity of NK cells against SCCHN cells supports a potential role for immune activation in variability of cetuximab-mediated clinical responses. Additionally, these polymorphisms correlated with NK cell activation and cytokine secretion. ADCC activity by PBMC from SCCHN patients and poor responder genotypes can be improved with IL-2 or IL-15 treatment. Serum cytokine levels, cellular immune profiles, or FcγR genotypes from patients' peripheral blood may provide clinically useful biomarkers of immune activation in cetuximab treated patients. Prospective clinical trials are necessary to validate these findings in SCCHN.

Immunocytokine KS-IL2 Increases Natural Killer (NK) Cell Immune Synapse Formation and Conjugates Effector and Target Cells Via the IL-2 Receptor

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Epithelial cell adhesion molecule (EpCAM) is overexpressed by a majority of ovarian tumors. The immunocytokine KS-IL2 is a fusion protein of 2 interleukin (IL)-2 molecules and the anti-EpCAM antibody, KS-1/4. KS-IL2 is currently being evaluated as a potential immunotherapeutic agent for the targeting of ovarian tumors. It is postulated that binding of KS-IL2 to the ovarian tumor cell surface will result in recruitment of natural killer (NK) and other cytotoxic cells via the Fc receptor (CD16). In addition IL-2 facilitates recruitment and activation of additional immune cells to the tumor microenvironment. Previous studies have shown that KS-IL2 enhances cytolytic function of NK cells derived from the peritoneal fluid and peripheral blood of ovarian cancer patients. In the current study we demonstrate that KS-IL2 facilitates immune synapse formation between the ovarian cancer cell lines, OVCAR-3 and NK cells derived from peripheral blood of healthy donors or ovarian cancer patients. Immune synapse formation facilitated by KS-IL2 is associated with polarization of LFA-1, CD2, F-actin, and perforin to the interface between NK and OVCAR-3 cells. An important factor to consider in immunotherapeutic strategies is the competence of the immune cells residing in the tumor microenvironment. NK cells residing in the peritoneal environment of ovarian cancer patients exhibit a severe down-regulation of CD16. Thus peritoneal NK cells may exhibit reduced ADCC with KS-IL2 treatment. It has previously been postulated, however, that KS-IL2 may facilitate NK cell-tumor cell conjugation via the IL-2 receptor. To address this possibility we utilized the CD16– NK leukemia cell line (NKL), cells that express high amounts of IL-2 receptor. Cell cytotoxicity experiments indicate that KS-IL2 significantly increases the ability of NKL cells to lyse OVCAR-3 targets. Flow cytometry-based cell conjugation experiments show that NKL cells form basal level of conjugates with OVCAR-3 cells. However, in the presence of KS-IL2, a 2.4-fold increase in conjugate formation is observed. This cell conjugation is inhibited by the anti-IL2 receptor antibody, Tac. In conclusion, we demonstrate that KS-IL2 facilitates immune synapse formation between NK and target cells. KS-IL2 also bridges NK and target cells via IL-2 receptor. This is the first demonstration that the IL-2 receptor, in addition to its immune activating functions, may also serve an important role in enhancing the immunocytokine response by serving as a cell adhesion molecule.

Phase I/II Study of CR011-vcMMAE, an Antibody-drug Conjugate Targeting GPNMB, for the Treatment of Patients With Advanced Melanoma

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Background: Glycoprotein NMB (GPNMB) is expressed by a number of tumor types including melanoma, breast cancer, and glioma, and has been shown to promote the invasion and metastasis of cancer cells. CR011-vcMMAE is a fully human monoclonal antibody directed against the extracellular domain of GPNMB conjugated to the tubulin-stabilizing agent monomethyl auristatin E (MMAE) via an enzyme-cleavable valine-citrulline (vc) linker. The antibody-drug conjugate is designed to bind GPNMB and undergo internalization, which leads to intracellular cleavage of the vc linker by endosomal proteases, releasing the cytotoxic MMAE.

Methods: The study objectives are to determine the safety, establish the maximum tolerated dose, and assess the activity of CR011-vcMMAE administered intravenously once every 3 weeks in patients with unresectable stage III or stage IV melanoma. Eligible patients have progressive disease at study entry and may have received prior cytokine, immune, or vaccine therapies, but no more than 1 prior cytotoxic therapy. Phase II uses a Simon 2-stage design with the primary endpoint of objective response rate.

Results: Thirty-two patients [26 (81%) stage IV; 11 M1b, 12 M1c] in phase I received doses of 0.03 to 2.63 mg/kg intravenous q3 weeks. Dose-limiting toxicities at 2.63 mg/kg were rash with desquamation (n = 2). The recommended phase II dose was 1.88 mg/kg intravenous q3 weeks. Preliminary adverse event data at this dose (n = 15) included: fatigue (n = 10), rash (n = 9, one grade 3), diarrhea (n = 8), and nausea (n = 8). Neutropenia was observed in 9 patients at 1.88 mg/kg; five were grade 2,

two were grade 3, and two were grade 4. Pharmacokinetic analysis showed terminal half-life for total antibody of 38 hours with less than 1% free MMAE. Tumor shrinkage, including one partial response by RECIST, was observed in phase I and appeared to be dose-dependent. In the ongoing phase II study, 18 patients (8 women; 10 men), median age 58 years (range, 38 to 70) were evaluable for response as of July 1, 2008. Three ongoing patients had partial response (1 confirmed, 2 unconfirmed); 12 patients had stable disease (median 9 wks, range 7+ to 20+ wk) with 10 continuing on study.

Conclusions: The antibody-drug conjugate CR011-vcMMAE is active and well tolerated in heavily pretreated patients with advanced melanoma. The phase II portion of the study has met the criteria for advancement into the second stage of accrual. Updated phase II data will be presented.

Radiofrequency Ablation With KS-IL2 Immunocytokine (EMD 273066) Results in an Enhanced Antitumor Effect Against Murine Colon Adenocarcinoma

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Background: Radiofrequency ablation (RFA) is a common treatment modality for surgically unresectable tumors. In this preclinical work, we sought to enhance the antitumor effect from RFA by adding immunotherapy in the form of the huKS-IL2 immunocytokine (EMD 273066) given to mice bearing CT26-KS colon adenocarcinoma. The huKS-IL2 immunocytokine (huKS-IL2) is an experimental immunotherapeutic reagent, composed of a humanized IgG1 antibody that detects the KS-antigen (an epitope on the human epithelial cell adhesion molecule), that is overexpressed on most epithelial carcinomas, including colon cancer.

Results: The addition of huKS-IL2 treatment to RFA-treated mice resulted in a significantly greater antitumor response as measured by suppression of tumor growth, compared to untreated animals and those treated with RFA or huKS-IL2 alone. Animals treated with huKS-IL2+RFA also had significantly enhanced survival compared to all other treatment groups. Further, after conditions were optimized, treatment with RFA+huKS-IL2 resulted in complete tumor resolution of established disease in 50% of mice, whereas under these conditions no mice in other groups resolved tumors. When immunological memory was tested in tumor-bearing mice that resolved smaller tumors, RFA+huKS-IL2 resulted in significantly more animals rejecting both CT26-KS and more aggressive CT26 tumors on rechallenge, compared to animals treated with RFA alone. This memory response was found to be tumor-specific, as animals, which previously rejected CT26-KS and CT26 did not reject an unrelated Meth A sarcoma. Treatment of a local tumor with RFA+huKS-IL2 also demonstrated antitumor effects against a distant untreated tumor. Flow cytometry analysis of T cells from mice from all treatment groups demonstrated that treatment with RFA+huKS-IL2 results in a greater proportion of cytokine producing (interferon and granulocyte macrophage-colony stimulating factor) CD4 T cells and CD8 T cells than all other treatment groups.

Conclusions: These results show that the addition of huKS-IL2 to RFA significantly enhances the antitumor response, resulting in complete tumor resolution and induction of immunological memory.

Cytokines Enhance the Anti-tumor Effects of Folate Conjugated Immunoglobulin

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Folate conjugation is a means to selectively target therapeutics to folate receptor (FR)-expressing cancer cells. A novel folate-bound immunoglobulin (F-IgG) was tested for its ability to target natural killer (NK) cells to folate-expressing cancer cells in the presence or absence of NK-activating cytokines. FR expression in cancer cell lines was confirmed by immunoblot analysis and flow cytometry. NK cells were tested for their ability to lyse FR-positive and FR-negative cell lines that were treated with F-IgG or C-IgG in the presence or absence of interleukin (IL)-2, IL-12, IL-15, and IL-21. NK cell production of interferon-γ was also quantitated. FR expression was confirmed in KB (human oral epithelial) and HeLa (human cervical cancer) cell lines. Binding of F-IgG to NK cell Fc receptors led to phosphorylation of the epidermal growth factor-related kinase. Lysis of KB tumor cells by NK cells was increased 3-fold to 8-fold following treatment with F-IgG as compared to C-IgG ($P < 0.0001$ across E:T ratios from 6.25:1 to 50:1). At the lowest E:T ratio, NK cell lysis of F-IgG–coated KB target cells was enhanced by 50% to 115% following treatment of NK cells with cytokines. NK cell production of interferon-γ in response to F-IgG–coated KB target cells was also significantly enhanced by IL-12 (P value = 0.005). The F-IgG construct did not bind to FR-negative cell lines (eg, A549 human alveolar basal epithelial) and did not enhance their lysis by NK cells. These studies suggest that F-IgG induces an immune response by NK cells against FR-positive cell lines and that cytokine treatment of NK cells will enhance this response.

ADCC-mediated Lysis of KRAS-mutated Colon Cancer Cells by Anti-EpCAM Antibody Adecatumumab

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Patients with mutations in the KRAS protooncogene have been shown to be largely resistant to therapy with the anti-EGFR monoclonal antibody cetuximab (Erbitux)® (Di Fiore F, et al. *Brit J Cancer*. 2007;96:1166). Cell culture experiments support this finding by showing that most KRAS-mutated colon cancer cell lines are refractory to the growth inhibitory activity of the antibody (Jhawer M, et al. *Cancer Res*. 2008;68:1953). Most resistant was colon cancer cell line HCT116, harboring a KRAS mutation in exon 2.

Here, we investigated the cytotoxic activity of adecatumumab (MT201) against a panel of human colon cancer cell lines, including cell lines HCT116, HT-29, and SW480. The assay investigated antibody-dependent cellular cytotoxicity (ADCC) as is mediated by recruitment of Fc-γ receptor-positive immune cells, largely natural killer cells. Adecatumumab recognizes epithelial cell adhesion molecule (EpCAM; CD326), which is highly and frequently expressed in >98% of colon cancer patients (Went P, et al. *Brit J Cancer*. 2006;94:128).

Adecatumumab showed high ADCC activity against all human colon cancer cell lines investigated. Although cetuximab was shown to potently exert ADCC in lung cancer cell lines (Kurai J, et al. *Clin Cancer Res*. 2007;13:1552), low ADCC activity was observed here with the anti-EGFR antibody against colon cancer cell line SW480, and essentially no ADCC activity against HT-29 and HCT119 colon cancer cells. This suggests that not only growth inhibition but also ADCC by cetuximab was suboptimal in KRAS-mutated (HCT116 and SW480) and even wild-type KRAS cancer line HT-29. By contrast, ADCC by adecatumumab was apparently not affected by KRAS mutations in colon cancer cells. Based on the high-level and frequent expression of EpCAM on colon cancers and the ADCC activity of adecatumumab against various colon cancer cell lines, we expect the human antibody to have potential for the treatment of patients with KRAS-mutated colon cancer.