Abstracts for the 27th Annual Scientific Meeting of the Society for Immunotherapy of Cancer (SITC)

(Presenting Authors are Italicized)

ADOPTIVE T CELL TRANSFER AND CELL THERAPY AS CANCER IMMUNOTHERAPY (CARS)

Adoptive Cell Therapy Using Expanded Autologous Tumor-infiltrating Lymphocytes in Metastatic Melanoma Patients: Role of Specific Lymphocyte Subsets

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Purpose: Adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) is a promising treatment for metastatic melanoma unresponsive to conventional therapies. We report here on the results of an ongoing Phase II clinical trial testing the efficacy of ACT using TIL in metastatic melanoma patients and the association of specific patient clinical characteristics and the phenotypic attributes of the infused TIL with clinical response.

Experimental Design: Altogether, 31 transiently lymphodepleted patients were treated with their expanded TIL followed by two cycles of high-dose (HD) IL-2 therapy. Persistence of infused TIL was tracked in the blood of patients at various time points after infusion using TCR Vβ cloning and CDR3 sequencing. The effects of patient clinical features and the phenotypes of the T-cells infused on clinical response were determined.

Results: Overall, 15/31 (48.4%) patients had an objective clinical response using immune-related response criteria (irRC), with two patients (6.5%) having a complete response. Progression-free survival of >12 months was observed for 9/15 (60%) of the responding patients. Discrete T cell clones from the infusion product were found at high frequency in the blood of responder patients up to 22 months post infusion. Factors significantly associated with objective tumor regression included a higher number of TIL infused, a higher proportion of CD8 + T-cells in the infusion product, a more differentiated effector phenotype of the CD8 + population and a higher frequency of CD8 + T-cells co-expressing the negative costimulation molecule "B- and T-lymphocyte attenuator" (BTLA). In an accompanying abstract presented at this meeting (Haymaker et al), evidence for the enhanced functional capacities of CD8 + BTLA + TIL is also shown.

Conclusion: These results indicate that immunotherapy with expanded autologous TIL can achieve durable clinical responses in metastatic melanoma patients. Infused T cells are capable of long term persistence post infusion. CD8 + T-cells in the infused TIL, particularly differentiated effectors cells and cells expressing BTLA, are associated with tumor regression.

Key Words: Cancer immunotherapy, Adoptive immunotherapy, Melanoma.

Role of the PD-1/PD-L1 Pathway on Regulatory T Cell Development, Induction and Function in vivo

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Regulatory T cells (Tregs) and the PD-1/PD-L1 pathway are important for the maintenance of peripheral tolerance. A subset of Tregs express PD-1 constitutively, suggesting a possible role for PD-1 in Treg biology. It has also been reported that PD-L1 promotes the induction of Treg in vitro. Based on these notions, we hypothesized that the PD-1/PD-L1 pathway may be important for Treg development, function and/or induction, and carried out a series of in vivo experiments to investigate this question. PD-1-/- mice harbored normal numbers of Tregs in the thymus and peripheral organs, and PD-1-/- Tregs developed normally in both polyclonal and Treg specific TCR transgenic bone marrow chimeras. PD-1-/- Tregs incorporated BrdU similarly to wildtype (WT) Tregs, suggesting that their in vivo Treg proliferation was normal. Compared to WT Treg, PD1-/- Treg expressed similar levels of Foxp3, CTLA-4, GITR and Helios and slightly lower levels of IL-2Rα (CD25). To assess the effect of PD-1 on Treg induction in vivo, naïve, polyclonal WT or PD-1-/- CD4 + FoxP3- T cells were adoptively-transferred into RAG2-/- mice, and were subsequently analyzed for FoxP3 expression. A significantly lower frequency of transferred PD-1-/- CD4 + T cells expressed FoxP3 compared to WT CD4 + T cells. Further, in an OVA oral tolerance model, induction of FoxP3 expression was markedly decreased among PD-1-/- OT-II versus WT OT-II CD4 + T cells. WT or PD-1-/- OT-II T cells were also CFSE-labeled prior to adoptive transfer and OVA challenge. In this setting, PD-1-/- OT-II T cells proliferated more vigorously in vivo, and the highest percentage of Treg conversion among WT OT-II T cells occurred in after 3-4 cell divisions, consistent with published in vitro data. These data suggest that decreased Treg induction of PD-1-/- OT-II T cells may be related to their enhanced proliferative capability. Lastly, the function of PD-1-/- Tregs was analyzed in a B16 melanoma model, where total or Treg-depleted splenic T cells from WT or PD-1-/- mice were transferred into tumor-bearing RAG2-/- mice. Depletion of PD1-/- Tregs from the total PD-1-/- T cell population did not further augment tumor rejection as was seen when WT Treg were depleted from WT Total T cells, suggesting that PD-1-/- Tregs may have a defect in suppressive capability. In conclusion, our results strongly suggest that the PD-1/PD-L1 pathway is important for the in vivo induction of Tregs, while PD-1 appears to be dispensable for natural Treg development. Whether PD-1 plays a role in Treg-mediated suppression is currently under investigation. These data raise the possibility that PD-1 blockade in cancer patients may function not only to re-activate effector T cells, but also to prevent the induction of Tregs.

Key Words: PD-1, Treg cells.

Phase I Study of Intraperitoneal Adoptive Cell Therapy With MHC Non-Restricted Tall-104 Cells in Patients With Peritoneal Carcinomatosis

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Purpose: TALL-104 is a human leukemic T cell line (CD3 + , CD4- CD8 + , CD56 + , CD16-, CD161 +) grown in IL-2, that has the ability to kill tumor cells in preclinical models in a MHC unrestricted way. In this Phase I study safety, immunological effects and pharmacodynamics of TALL-104 cells given as intraperitoneal (IP) infusion in patients with peritoneal carcinomatosis were assessed.
Experimental design: Fifteen patients with peritoneal carcinomatosis from gastrointestinal (GI, 7 patients) or ovarian cancer (OC, 8 patients) not amenable for surgery, age between 18 and 75 years, performance status (PS) ≤ 2 (ECOG scale) and a life expectancy > 6 months were included in the study. Irradiated TALL-104 cells were administered, as IP infusions, on day 1, 3, 5, 15 and 30 using a cell escalation design. Starting dose was 1×10^8 cells/infusion; subsequent dose levels were 5×10^8 and 2.5×10^9 cells/infusion. Primary study objective was safety; secondary objectives were the kinetics of TALL-104 cells in ascitic fluid (AF) and peripheral blood, levels of cytokines dosed in AF and serum, immunological monitoring and clinical outcome.
Results: Five patients have been treated at each dose level for a total of fifteen patients: 8 with OC and 7 with GI cancer. No treatment-related serious adverse events were observed and no significant toxicity was associated with TALL-104 infusions. The presence of TALL-104 in AF was detected at 24 and 48 hours after infusion in 12 and 3 samples respectively. Cytotoxicity of autologous mononuclear cells showed a mean increase up to 5% at day 3 in patients treated with 1st and 2nd dose level; HLA-DR + / CD14 + cells showed a mean increase up to 5% at day 3 through day 15 in all patients. IL-10, sICAM and sIL-2R in serum, showed a remarkable decrease at 2nd dose level. In AF an overall decrease of HGF, TGF-β, IL-10, sICAM1, sIL-2R and an acute increase of TNF-α at the 2nd cell dose level was observed. Six and 5 patients had a confirmed stable disease at day 45 and 90 respectively, with a median duration of 44 days (12-210).
Conclusion: TALL 104 cells administered by IP route showed a very good safety profile and doses above 5×10^8 cell/infusion are likely the recommended doses for a phase II trial. Cytokine levels, immunological parameters, and preliminary clinical findings suggest a potential antitumor effect of TALL 104 as adjunctive therapy.
Key Words: Adoptive therapy, CD8 + T cells, Ovarian cancer.

Gamma Delta T Cells: Natural Tumor Killers Amplified By Chimeric Antigen Receptors

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Chimeric Antigen Receptors (CARs) and gamma delta T-cells have demonstrated clinical efficacy as cancer therapies independently of one another. CAR binding directly to tumor antigens (Ag), e.g. CD19 on B-cell leukemia, activates CAR intracellular domains leading to tumor killing and growth of CAR $^+$ T cells independent of their T-cell Receptor (TCR) specificity. Gamma delta T-cells can be identified by the variable (V) region of their TCR, where V(delta)1 and V(delta)2 subsets have independently demonstrated anti-tumor immunity, but adoptive T-cell therapy is currently limited to V(delta)2 because of limited expansion methods. We hypothesized that a CAR would expand both V(delta)1 and V(delta)2 gamma delta T-cells independent of their TCR and would thus re-direct their killing abilities to Ag $^+$ tumors. The ability of gamma delta T-cells to grow on Ag $^+$ artificial antigen presenting cells (aAPC) without the CAR was first evaluated. Co-cultures were set up with aAPC, exogenous administration of interleukin-2 and -21 (IL-2 and IL-21), and paramagnetic bead-sorted gamma delta T-cells from peripheral blood mononuclear cells (PBMC). Unexpectedly, gamma delta T-cells proliferated on aAPC at a rate of greater than 10-fold increases per weekly stimulation, and this phenomenon was cytokine (IL-21) and CD86/ 41BBL co-stimulation dependent. Expanded gamma delta T-cells were comprised of V(delta)1 and V(delta)2 subsets, which displayed broad anti-tumor capabilities against a number of tumor cell lines from leukemia (B- and T-cell), colon, pancreatic, and ovarian cancer but did not lyse normal allogeneic B-cells. Killing abilities of these gamma delta T-cells was then re-directed with CD19-specific CAR. Sleeping Beauty transposase and a CAR transposon were electroporated into PBMC to establish stable CAR expression in T-cells, and paramagnetic bead sorting was used to isolate gamma delta T-cells the day after electroporation. CAR $^+$ T-cells were propagated on CD19 $^+$ aAPC and yielded over 10^9 CAR $^+$ T-cells (> 103 fold change) after a month of culture. Both V(delta)1 and V(delta)2 subsets were present at high frequencies in CAR + T-cells, which displayed enhanced killing of Ag + tumor cell lines in vitro compared to gamma delta T-cells not expressing CAR. Tumor xenografts in immunocompromised mice were significantly eliminated when treated with CAR + gamma delta T-cells compared to mock treated mice. In sum, both V(delta)1 and V(delta)2 gamma delta T-cells expanded robustly on aAPC and their inherent killing abilities could be amplified through CAR expression. This study bridged two adoptive T-cell therapy approaches that had shown efficacy separately and portrays potential for clinical translation.
Key Words: T cells, Cancer immunotherapy, Chimeric receptors.

Selection of PD-1, LAG-3, TIM-3 and 41BB Positive CD8 T Cells in the Fresh Tumor Digest Enriches for Melanoma-Reactive Cells

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Tumor reactive T cells can be found infiltrating melanoma lesions. However, the isolation of T cells specific for tumor antigens infiltrating these lesions has remained a challenge since there are no phenotypic parameters that can be used to consistently identify them. PD-1, LAG-3 and TIM-3 are some of the negative co-stimulatory molecules that have been proposed to be expressed on tumor-reactive T cells as a result of chronic antigen stimulation. 41BB, in the other hand, is a positive co-stimulatory molecule which is up-regulated on CD8 T cells upon TCR engagement. The main objective of our work was to characterize the phenotype of TIL in fresh melanoma tumor digests and to test whether any of the markers studied could be used to enrich for tumor-specific cells. We first studied the expression of PD-1, LAG-3, TIM-3 and 41BB on CD8 T cells in fresh melanoma tumor digests, as well as their stage of differentiation (CD62L, CCR7, CD45RO, CD27, CD28 and CD57). We found that CD8 + cells in melanoma tumors were enriched in effector memory-like cells (CD62L- CD45RO +) compared to peripheral blood. CD8 + tumor infiltrating lymphocytes showed higher frequencies of TIM-3 (15%), PD-1 (13%),LAG-3 (8%) and 41BB (2%) expression compared to peripheral blood. By using a Mart-1 peptide-MHC tetramer to analyze the phenotype of cells with specificity for a known melanoma antigen, we observed that these cells displayed an effector memory-like phenotype (CD62L-, CCR7-, CD45RO +) and higher levels of PD-1, LAG-3 and TIM-3 than the tetramer negative population. Furthermore, a subset of TIL co-expressed TIM-3, PD-1 and LAG-3 consistent with the presence of an exhausted phenotype in a subpopulation of CD8 TIL and suggesting some of these markers might be useful for enriching melanoma-reactive cells. Most importantly, we performed functional experiments in which we separated distinct T cell populations present in the fresh tumor digest according to expression of the phenotypic markers studied, expanded them in vitro and tested the reactivity of these populations against their autologous tumor cell lines. Tumor-reactivity was found preferentially in effector cells derived from the cells expressing PD-1, LAG-3, TIM-3 and 41BB in the fresh tumor digest but not in the cells lacking the expression of these markers. Positive selection of cells expressing PD-1, LAG-3, TIM-3 and 41BB resulted in a considerable enrichment of tumor reactive cells

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compared to the bulk CD8 T cells expanded from the fresh tumor digest. Our results suggest that tumor-reactive T cells in fresh melanoma digests express PD-1, LAG-3, Tim-3 and 41BB and thus, these markers can be used to enrich for melanoma-reactive cells.

Key Words: Melanoma immunotherapy, Adoptive immunotherapy, Tumor infiltration lymphocytes.

γ9- and δ2-CDR3 Domains Regulate Functional Avidity of T Cells Harboring γ9δ2 T Cell Receptors

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Immunotherapy with innate immune cells has recently evoked a broad interest as a novel treatment option for cancer patients. γ9δ2T cells are an emerging innate cell population with strong anti-tumor reactivity, which makes them a promising candidate for immune interventions. γ9δ2T cell receptors (TCR) in particular recognize a broad panel of tumor cells with high avidity, and are therefore clinically attractive, since αβT cells can be efficiently redirected against a variety of tumor cells by introducing a γ9δ2TCR. Here we demonstrate that distinct γ9δ2TCRs mediate different functional avidities, and present the concept of combinatorial-γδTCR-chain-exchange (CTE) as an efficient method to create γ9δ2TCRs that mediate strong anti-tumor-responses. In this way, γ9- and δ2-chains derived from individual γ9δ2T cell clones are newly combined, allowing the design of γ9δ2TCRs that mediate a significant higher functional avidity against a broad tumor cell panel in vitro and in vivo when compared to a reference γ9δ2TCR. In addition, we demonstrate that this phenomenon is selectively caused by differences in the CDR3 domains of γ9- and δ2-chain. Accordingly, an alanine-scanning-mutagenesis was performed to elucidate important residues within the CDR3 sequence and the impact of the CDR3 length for optimal γ9δ2TCR function. While length and sequence seem to both play critical roles in δ2-CDR3, selectively the γ9-CDR3 sequence but not the length is a crucial factor. To summarize, structurally and functionally important residues within the CDR3 domains of a γ9δ2TCR were identified, suggesting a thus far underestimated role of δ2-CDR3 in particular in antigen-recognition. This knowledge allowed improved tumor control by using engineered T cells, not only in vitro, but also in vivo in a humanized mouse model.

Key Words: TCR, T cells, Adoptive immunotherapy.

Targeting CD22 Expressing B Cell Leukemia With Chimeric Antigen Receptors (CAR): Engineering Membrane Proximity and Second Signaling Motifs for Optimal Activity

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CD22 is expressed on the surface of B cell hematologic malignancies such as acute lymphoblastic leukemia (ALL) and also expressed on normal B cells. CD22 is a Siglec family lectin present on B cells, starting at the pre-B cell stage of development, but is not expressed on plasma cells. CD22 consists of 7 extracellular Ig domains and is found in 2 isoforms one of which is missing the second and third N-terminal Ig domains. We generated CAR modified T cells containing anti-CD22 extracellular binding motifs and intracellular signaling domains for T cells activation (CD3 zeta) or costimulation (CD28 or 4-1BB). To find the optimal CD22 CAR we investigated 2 scFvs against CD22. One binding domain is derived from the HA22 immunotoxin (Moxetumomab pasudodtox) and binds the third Ig domain of CD22. We have also developed CD22 CARs that bind a membrane proximal CD22 domain (binding between domains 5-7), derived from the m972 fully human high affinity monoclonal antibody generated by phage display (Xiao et. al mAbs. 2009). To evaluate the potency of activation and persistence of CD22 CARs we generated second-generation constructs (CD28 and CD3 zeta; or, 4-1BB and CD3 zeta) and a third generation construct (containing CD28, 4-1BB and CD3 zeta domains). In vitro cellular cytotoxicity experiments with 4 B cell-ALL cell lines revealed that second generation CD22 CARs expressing CD28 or 4-1BB were significantly better in lytic assays than third generation vectors. In vitro proliferation experiments are currently being evaluated using different methods of initial T-cell activation (OKT3 or anti-CD3 anti-CD28 beads) in order to determine which CAR-expressing T cells better expand in an antigen-dependent manner upon subsequent re-stimulation. We are evaluating anti-CD22 CAR activity in vivo using a pre-B-ALL xenograft mouse model, i.e. the NALM6-GL cell line, which stably expresses luciferase. NSG mice were injected i.v. with 0.5E6 NALM6-GL, three days later the mice were treated with 1E7 CAR + T cells, and then followed by bioluminescent imaging to measure disease burden. Preliminary data indicate that the HA22 second generation with CD28 is more potent at tumor clearance than 4-1BB, and that both are more potent than third generation vectors. Further definition of CAR-CD22 interactions and of T cell activation mediated by differing CAR signaling formats will guide future pre-clinical models for anti-leukemia immunotherapy.

Key Words: Adoptive immunotherapy, B-ALL, Chimeric receptors.

BTLA: New Biomarker for a Highly Proliferative CD8 + TIL Subset Associated With Melanoma Regression During Adoptive Cell Therapy

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Adoptive T cell therapy using tumor-infiltrating lymphocytes (TIL) expanded ex vivo with high-dose IL-2 is a promising approach for the treatment of metastatic melanoma. Recently, our lab demonstrated the importance of CD8 + T cells expressing B and T lymphocyte attentuator (BTLA) with a positive clinical response. This suggests that functional differences may exist between CD8 + BTLA + and CD8 + BTLA- cells resulting in the differential therapeutic potency of TIL in treated patients. Here, we isolated BTLA + and BTLA- CD8 T cell subsets from expanded TIL from metastatic melanoma patients accrued in a Phase II clinical trial at MD Anderson and performed functional assays measuring proliferation, apoptosis, cytokine production, and CTL activity as well as differences in global gene expression profiles between the subsets using microarray analysis. Functional analysis revealed that CD8 + BTLA + TIL exhibited superior proliferative capacity compared to CD8 + BTLA- TIL in response to IL-2 and anti-CD3/CD28 stimulation correlating with a higher degree of IL-2-induced STAT5 activation. CD8 + BTLA + TIL also had increased baseline levels of major cell cycle proteins such as cyclin B1 and CDK1 by reverse phase protein array analysis. Notably, we also found that CD25 was mainly expressed in CD8 + BTLA + T cells lacking PD-1 expression. CD8 + BTLA + TIL produced higher levels of IFN-γ, TNF-α, IL-2, and MIP-1β but did not have higher levels of CTL activity against OKT3-loaded targets or autologous tumor lines. However, ligation of BTLA with HVEM-Fc/anti-CD3 did reduce CD8 + BTLA + proliferation. Finally, our microarray analysis revealed significant differences in gene expression between CD8 + BTLA + and CD8 + BTLA- TIL, including higher expression of CD28 and IL-7R in the BTLA + subset. Interestingly, NK-associated markers in the KIR family were highly enriched in the BTLA- subset suggesting a more late-stage phenotype. These results suggest that BTLA is a new and

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powerful biomarker during T cell therapy for metastatic melanoma. Overall, BTLA appears to be a critical marker distinguishing a less differentiated, more highly active and polyfunctional CD8 + T cell subset with high responsiveness to IL-2. As BTLA ligation by its binding partner HVEM (which is expressed on melanoma cells) results in a decreased proliferative responsiveness to anti-CD3 stimulation, paradoxically, BTLA signaling however may still serve as a negative co-inhibitory molecule. Our results also underscore that increased expression of T cell co-inhibitory molecules may not be necessarily markers of "exhaustion", but instead be markers of more highly-activated, responsive T cells susceptible to negative regulation.

Key Words: Adoptive immunotherapy, CD8 + T cells, Melanoma.

Hematopoietic Stem Cell Characterization With a 19F Tracer Agent; The Ability to Evaluate Cellular Persistence

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Hematopoietic stem cells (HSC) have numerous applications including immune reconstitution, enzyme replacement, regenerative medicine and immunomodulation. The trafficking and persistence of these cells after administration is a question fundamental to the therapeutic applications of HSC. Here we describe the labeling of human CD34 + HSC with a perfluorocarbon (19F) tracer agent and address their detection in vivo. A comparison of unlabeled and 19F-labeled human CD34 + bone marrow isolates demonstrates the maintenance of therapeutic efficacy in both hematopoietic reconstitution and self-renewal studies. The lack of interference in these highly complex biological processes both in vitro and in vivo following 19F labeling provides strong evidence that the therapeutic potential of the HSC is likely to be maintained. Pilot studies to visualize HSC application address both intramuscular injection and cellular scaffold supporting implantation and demonstrate the importance of cellular persistence studies. Direct injection of cells resulted in a dissipation of the cells that proved difficult to analyze. Implementing a cellular scaffold allowed for the persistence of the HSC application. Migration and homing studies are currently being addressed. These data support the safety and utility of using PFC tracers for clinical applications of HSC and the assessment of cellular persistence.

Key Words: Cell trafficking, Targeted therapeutics.

Targeting Microsatellite Instable Colon Cancer With Adoptively Transferred Redirected T cells

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Adoptive transfer of genetically engineered T cells is a promising immunotherapeutic approach for the treatment of cancer. However, recent findings both in the clinic and in pre-clinical mouse models indicate that careful consideration of the target antigen should be made to avoid on-target toxicity. Rather than targeting tumour-associated auto-antigens, it may be safer directing engineered T cells against mutated proteins such as frequently occurring frameshift mutations. These mutations result in polypeptides which are not otherwise available for antigen processing and are thus truly tumour specific. The exquisite tumour specificity of such mutations also avoids the problem of low affinity TCRs due to central tolerance. One such example is the transforming growth factor β Receptor II (TGFβRII) frameshift mutation found in hereditary non-polyposis colorectal cancers (HNPCC) and around 15% of sporadic colorectal and gastric cancers displaying microsatellite instability (MSI). The -1A mutation in an adenine stretch of the TGFβRII gene gives rise to immunogenic peptides which have previously been used for vaccination of MSI + colorectal cancer patients in a Phase I clinical trial. In one of these patients, we identified and cloned a novel HLA-A2-restricted TGFβRII frameshift mutation-specific TCR (TGFβRII-TCR) from a CD8-/CD4- CTL clone.

Cloning and expression of TGFβRII-TCR in Jurkat cells showed that this TCR was co-receptor independent, but HLA-A2/peptide-specific. Consequently, electroporation of mRNA encoding the TGFβRII-TCR into polyclonal, in vitro expanded human T cells showed that both CD8 + and CD4 + T could be redirected against HLA-A2/TGFβRII peptide expressing cells. Indeed, cells expressing the TGFβRII-TCR were functional following recognition of peptide-loaded HLA-A2 positive target cells as well as colon carcinoma cell lines harbouring the mutation.

Transient TCR expression may also be a safer alternative compared with stable gene expression for the first evaluation of a novel TCR in the clinic, but requires multiple T-cell infusions to compensate for the short-lasting transgene expression.

We performed pilot studies in a murine model of MSI + colorectal cancer which indicated that the colon cancer cell line HCT-116 engrafted well and that adoptively transferred redirected TGFβRII-T cells homed to the tumour and displayed anti-tumour activity. Alloreactivity in our control, mock-electroporated T cells complicated the interpretation of the results and we are now further optimising in vivo experiments to demonstrate the therapeutic potential of TGFβRII-TCR expressing T cells.

Key Words: TCR, Adoptive immunotherapy, Animal model.

MIR155 Augments the Anti-Tumor Activity of CD8 + T Cells by Enhancing Responsiveness to Homeostatic Cytokines in the Absence of Lymphodepletion

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Lymphodepleting preconditioning regimens are routinely employed to remove endogenous cellular sinks for homeostatic cytokines thus augmenting the engraftment and anti-tumor efficacy of transferred tumor-reactive T cells. We found that self/tumor-specific CD8 + T cells constitutively expressing miR155, a microRNA highly expressed in effector and memory T cells, displayed enhanced proliferation and anti-tumor function in the absence of lymphodepletion. The benefit of miR155-transduced T cells compared to control cells expressing a scrambled miR was minimized in irradiated or genetically lymphodepleted hosts, suggesting that miR155 enhances T cell activity only under conditions of limited homeostatic cytokines availability. Conversely, the increased functionality of miR155 overexpressing CD8 + T cells was virtually abrogated in mice deficient in the homeostatic cytokine interleukin-15. We found that miR155 inhibited the expression of several negative regulators of signal transducer and activator of transcription (STAT) signaling, including the protein tyrosine phosphatase Ptpn2. Consistently, STAT5 phosphorylation in response to homeostatic cytokine stimulation was enhanced in miR155-transduced CD8 + T cells while expression of a constitutive active Stat5a variant recapitulated the functional advantage conferred by miR155. Thus, miR155 augments the anti-tumor activity of CD8 + T cells by acting as a STAT5 rheostat to facilitate signaling when homeostatic cytokines are limiting. These findings indicate that miR155 might be employed to enhance the effectiveness of adoptive immunotherapies in a cell intrinsic manner without the need of potentially life-threatening, lymphodepleting maneuvers.

Key Words: Engineering, Adoptive immunotherapy, CD8 + T cells.

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On a Critical Role for IL2 Adjunctive Co-Therapy for Successful Suppression of Solid Tumor With Designer T Cells

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Introduction: We created chimeric antigen receptors (CAR) specific for prostate specific membrane antigen (PSMA). When expressed in patient T cells, these "designer T cells" (dTc) specifically kill prostate cancer cells in vitro and in vivo in animal models, with 5/9 (55%) of xenografted mice experiencing complete remissions (Ma et al Prostate 2004:61:12-25). A Phase I clinical trial was approved by the FDA.

Methods: Patient T cells are retrovirally transduced and expanded ex vivo to span dose levels of 10ˆ9 to 10ˆ10 T cells. Patients undergo prior non-myeloablative (NMA) conditioning to create a "hematologic space" into which designer T cells are infused for stable engraftment and prolonged in vivo efficacy. Patients are co-administered continuous infusion low dose IL2 (LDI: 75 kiu/kg/d) for 28 days.

Results: Five patients were treated, three at 10ˆ9 and two at 10ˆ10 cell dose levels. Patients experienced neutropenic fever after conditioning, but no designer T cell-related toxicities. Partial responses (PR) in 2/5 patients (40%) with metastatic cancer, with PSA suppressions of 50–70% over 1–2 months and PSA progression delay of up to 150 days. Yet these responses were observed only at the lowest T cell dose (10ˆ9 cells) and not at the higher tested dose (10ˆ10 cells). Response was found to correlate ($P = 0.03$) with the levels of IL2 in the plasma that were as much as 10-fold lower in the non-responders versus the responders. This lower level of IL2 correlated in turn with higher engrafted fractions of infused activated T cells ($P = 0.03$). This prompted the hypothesis that the infused activated T cells at high engrafted cell numbers were absorbing out IL2 to a level too low to sustain dTc activation for effective tumor killing. A study redesign will test moderate dose IL2 (MDI) (300 kiu/kg/8 h bolus) versus low dose IL2 (LDI) in a randomized Pilot study at the 10ˆ10 cell dose.

Conclusion: An application of designer T cells in adoptive immune therapy of advanced prostate cancer has had encouraging early results. It is proposed that adequate higher IL2 levels in vivo could allow the greater potency of higher dTc doses to be revealed, thereby potentially inducing PSA reductions of 100%, with durable remissions of metastatic prostate cancer that is refractory to all other treatments. Patients are being actively recruited. For patient referrals, please contact by telephone: (401) 456-2507 or email: RDavies@rwmc.org. This clinical trial received partial funding from the US Army/DOD. Preclinical work was supported by the Prostate Cancer Foundation.

Key Words: T cells, Prostate cancer, Immunotherapy.

Cytokines in the IL-12 Family Distinctly Impact the Functional Fate and Anti-Tumor Activity of TC17 Cells

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IL-17 secreting CD8 + T (Tc17) cells play an important role in regulating infectious diseases, cancer and autoimmune disorders, but how distinct inflammatory cytokines regulate their long-term function and persistence remains unknown. Given that cytokines in the IL-12 family, such as IL-12, IL-23, and IL-27, impact the function of Tc17 cells in vitro, we sought to investigate how these IL-12 family cytokines regulate Tc17 cell-mediated tumor immunity. To address this question, Tc17-polarized Pmel-1 CD8 + T cells specific for melanoma-associated antigen, glycoprotein (gp)100 were primed with IL-12 (Tc17 + IL-12), IL-23 (Tc17 + IL-23) or IL-27 (Tc17 + IL-27). Intracellular cytokine staining and ELISA revealed that Tc17 cells primed with IL-12 have increased polyfunctionality and produced heightened levels of IL-17, IFN-γ, TNF-α and IL-10 compared to Tc17 cells primed with IL-23 or IL-27. In contrast, no differences in function by Tc1 cells were observed when they were primed with IL-12, IL-23 or IL-27. Tc17 cells primed with IL-12 also expressed elevated levels of CD25 and ICOS. To understand how these functional and phenotypic differences affect the anti-tumor activity of the Tc17 cells, we adoptively transferred Tc17 cells primed with IL-12, IL-23 or IL-27 into a lymphodepleted C57BL/6 host bearing B16F10 melanoma. Interestingly, we found that Tc17 cells primed with IL-12 or IL-27 mediated superior tumor regression compared to those primed with IL-23.

Furthermore, IL-12 primed Tc17 cells persisted in vivo superior to Tc17 cells primed with IL-23 or IL-27. Thus, IL-12, IL-23 and IL-27 differentially regulate the functional fate and anti-tumor activity of Tc17 cells. Unraveling how IL-12 and IL-27 endow Tc17 cells with abiding memory to tumors will have immediate relevance for the treatment of cancer patients. As the proposed studies focus on determining the specific means by which cytokines in the IL-12 family augment Tc17 cell-mediated tumor immunity, this work will have broad clinical significance for understanding and harnessing Tc17 (and Th17) cell memory to tumor antigens expressed on many advanced malignancies.

Key Words: Melanoma immunotherapy, Tumor immunity, Adoptive immunotherapy.

Growth and Activation of Natural Killer Cells ex vivo From Children With Neuroblastoma for Adoptive Cell Therapy

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Purpose: Adoptive transfer of natural killer (NK) cells combined with tumor-specific monoclonal antibodies (mAbs) has therapeutic potential for malignancies. We determined if large numbers of activated NK (aNK) cells can be grown ex vivo from peripheral blood mononuclear cells (PBMC) of children with high-risk neuroblastoma using artificial antigen-presenting cells (aAPC).

Experimental Design: Irradiated K562-derived Clone 9.mbIL21 aAPC were co-cultured with PBMC, and propagated NK cells were characterized with flow cytometry, cytotoxicity assays, Luminex® multi-cytokine assays, and a NOD/SCID mouse model of disseminated neuroblastoma.

Results: Co-culturing patient PBMC with aAPC for 14 days induced 2363 ± 443-fold expansion of CD56 + CD3-CD14- NK cells with 83 ± 4% purity (n = 10). Results were similar with PBMC from normal donors (n = 5). Expression of DNAM-1, NKG2D, FcγRIII/CD16 and CD56 increased 6 ± 3, 10 ± 2, 21 ± 20, and 18 ± 3-fold respectively on day 14 of co-culture compared to day 0, demonstrating activation of NK cells. In vitro, aNK cells were highly cytotoxic against neuroblastoma cell lines, and killing was enhanced with GD2-specific monoclonal antibody ch14.18. When mediating cytotoxicity with ch14.18, release of TNFα, GM-CSF, IFNγ, sCD40L, CCL2/MCP-1, CXCL9/MIG, and CXCL11/I-TAC by aNK cells increased 4-, 5- 6-, 15-, 265-, 917- and 363-fold (151-9,121 pg/mL), respectively, compared to aNK cells alone. Survival of NOD/SCID mice bearing disseminated neuroblastoma improved when treated with thawed and immediately intravenously infused cryopreserved aNK cells compared to un-treated mice and was further improved when ch14.18 was added.

Conclusion: Propagation of large numbers of aNK cells that maintain potent anti-neuroblastoma activities when cryopreserved supports clinical testing of adoptive cell therapy with ch14.18.

Key Words: Neuroblastoma, NK cells, Immunotherapy.

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Evaluating the Susceptibility of Solid Tumors to Chimeric Antigen Receptor Modified T Cell Therapies

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Adoptive cell therapy with tumor infiltrating lymphocytes (TIL) has been successfully used as a treatment in metastatic melanoma. However, TIL are not present in the majority of solid cancers. To overcome this barrier, T cells can be transduced with chimeric antigen receptors (CARs) to produce tumor specific T cells for adoptive immunotherapy. Anti-CD19 CAR T cell therapies have been effectively used to treat B cell hematologic malignancies in both pre-clinical and clinical studies, but we have observed less success when targeting solid tumors with CAR therapies in pre-clinical models. We hypothesize that this relates to a more hostile microenvironment within solid tumors compared to liquid tumors. To normalize for potential differences in tumor antigens, we created a CD19 expressing osteosarcoma cell line, thus allowing use of the well-characterized CD19 CAR to explore relative susceptibilities of solid versus hematologic malignancies to adoptive immunotherapy.

The 143B human osteosarcoma cell line was transfected to express CD19 (143B-CD19). In vitro 51-Cr-release assays demonstrated that CD19 CAR T cells had strong cytolytic activity against 143B-CD19. In vivo susceptibility of 143B-CD19 versus NALM6 (a CD19 + B-ALL line) was then assessed using a xenograft model. NSG mice were injected IM with 5e5 143B, 143B-CD19, or luciferase expressing NALM6. On day 3, mice were treated IV with 5e6 CD19-CAR or mock T cells. In four of five IM-injected NALM6 mice, CD19 CAR T cell treatment cleared all evidence of tumor as assessed by bioluminescence, and provided a survival benefit exceeding 60 days compared to controls ($P < 0.01$). However in 143B-CD19 injected mice, CD19 CAR T cell treatment failed to eradicate any tumors and only prolonged survival by an average of 23 days ($P < 0.01$). Further, we observed that human solid tumors expand murine myeloid derived suppressor cells (MDSCs) in this system, which bear a hallmark phenotype and suppress human T cells. Our ongoing work focuses on assessing the in vivo contribution of MDSCs to solid tumor resistance to CAR therapy. We conclude that solid tumors are less susceptible to CAR based therapy, even when expressing optimal tumor antigens, and hypothesize that MDSCs may be in part responsible for immune evasion of solid tumors in this model system.

Key Words: Cancer immunotherapy, Adoptive immunotherapy, Chimeric receptors.

Cytotoxic T Cells Expressing GUCY2C-Specific Chimeric Antigen Receptor as Targeted Therapy for Metastatic Colorectal Cancer

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Adoptive T cell therapy (ACT) has been successful in early phase clinical trials for the treatment of metastatic melanoma and B cell leukemia. In that context, advances in genetic engineering enable introduction of antigen-specific receptors into peripheral blood immune cells to generate large numbers of cytotoxic T cells ex vivo that are specifically targeted to tumors. Moreover, chimeric antigen receptors (CARs) that possess an antigen recognition domain derived from antibody variable regions coupled to cytoplasmic T cell receptor signaling domains produce cytotoxic immune cells that recognize and destroy their targets independently of T cell receptor-MHC interactions. Colorectal cancer is the fourth most commonly diagnosed and second leading cause of cancer related deaths in the United States. Mortality reflects metastatic disease associated with advanced stage: five-year survival rates drop from 90% in localized disease to 12% in patients with distant metastases. To date, ACT in colorectal cancer has been limited by antigen-targeted toxicities resulting in severe colitis and one patient death using cells targeting CEA and Her2 respectively, demonstrating the unmet need for antigens that better discriminate tumor from normal tissues. Guanylyl cyclase C (GUCY2C) is a membrane-bound cyclase expressed selectively on apical surfaces of intestinal epithelial cells. Further, GUCY2C expression is maintained throughout colorectal tumorigenesis, with universal (> 95%) expression by metastatic colorectal cancer. Luminal expression by epithelial cells sequesters GUCY2C from the systemic compartment by tight junctions forming the mucosal barrier, providing a unique opportunity to target systemic metastases without damaging GUCY2C-expressing intestinal epithelium. Here, we describe the application of retroviral transduction to express GUCY2C-specific CARs in mouse T cells. In vitro these CARs induce polyfunctional T cell cytokine production and direct anti-tumor cytolysis in an antigen-dependent manner. Moreover, GUCY2C-specific CAR T cells effectively eliminate pulmonary metastases of colorectal cancer in mice in vivo. These data suggest that cytotoxic T cells expressing GUCY2C-specific CARs may offer a novel immunotherapeutic approach to patients with metastatic colorectal cancer.

Key Words: Colorectal cancer, Adoptive immunotherapy, Chimeric receptors.

Inducible Costimulator (ICOS) Augments the Antitumor Activity of TC17 Cells

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IL-17-producing CD8 + T cells, called Tc17 cells, have been identified in both mice and humans, but their role in regulating immunity to tumor tissue remain incompletely elucidated. Tc17 cells have recently been discovered to exhibit potent antitumor immunity in mice and have been shown to possess enhanced memory properties. The cytokines that program CD8 + T cells towards a Tc17 cell phenotype have been identified, but the costimulatory molecules important for regulating their function and phenotypic fate remain unknown. We discovered that the inducible costimulator ICOS (CD278) is critical for augmenting the antitumor activity IL-17-producing CD8 + T cells. In this study, we examined the role of ICOS using a clinically relevant pmel-1 adoptive cell transfer therapy model for murine melanoma. We found that adoptively transferred ICOS + Tc17 cells secreted significantly more IL-17 on day 3 post-transfer compared to ICOS-Tc17 cells. In comparison to ICOS + Tc1 cells, ICOS + Tc17 cells secreted a similar amount of IFN-γ on day 3, but ICOS + Tc17 cells continued to secrete heightened amounts of IFN-γ at day 28. Additionally, blocking ICOS signaling by using ICOSL$^{-/-}$ mice or by using an anti-ICOS antibody (20 μg/mL days 2, 4, and 6 in culture) dramatically impaired Tc17 cell-mediated tumor destruction. Conversely, activating Tc17 cells with an ICOS agonist augmented their polyfunctionality, thereby improving their capacity to eradicate large tumors. This was associated with increased expression of ICOS, IL-2Rα, and IL-7Rα expression on Tc17 cells. To uncover the ideal signal(s) to generate human Tc17 cells for clinical use, antigen-specific human Tc17 cells were expanded with K562 artificial APCs (aAPCs). We used aAPCs expressing ligands for the T cell receptor (CD3), ICOS and/or CD28 to expand human Tc17 cells.

Interestingly, ICOS stimulation endowed Tc17 cells with superior multifunctionality and improved antigen-specific lytic ability compared to those stimulated with CD28. Our data shows that ICOS bolsters the function and persistence of murine Tc17 cells in a mouse model of melanoma and also potentiates the function and

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lytic capacity of human Th17 cell function. Collectively, our data reveal that targeting the ICOS/ICOS ligand pathway may have therapeutic merit for cellular therapy for patients with advanced malignancies and have broad clinical implications for the design of next generation vaccine and cellular therapies. Methods: Recipient mice, bearing established B16F10 melanoma, were pretreated with 5 Gy TBI. Mice then received 1e7 pmel-1 CD8 + *in vitro*-vaccinated Tc1 or Tc17 cells in conjunction with bolus IL-2. 5-8 mice were used per treatment group and all experiments were repeated. Human studies are representative of three experiments.
Key Words: Adoptive immunotherapy, CD8 + T cells, Melanoma.

Immunotherapy of Cancer: Reprogramming Tumor-Immune Crosstalk

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The inability of the host's immune response to eliminate tumor cells results from: i) the expression of weakly immunogenic tumor antigens coupled with a low frequency and low affinity of T cells, and ii) the suppression of anti-tumor immune responses. Therefore, we propose to induce fundamental changes in the tumor as well as immune cells in order to establish a new tumor/immune interaction that dominates the tumor with an effective immune response. We hypothesized that epigenetic modulation of tumor cells in situ using decitabine (Dec), a DNA demethylating agent, can render breast cancer more immunogenic by inducing the expression of cancer/testis antigens (CTA). However, the accumulation of myeloid-derived suppressor cells (MDSCs) may inhibit rejection of established tumors mediated by CTA-reactive immune cells. Therefore, we developed the ability to re-program CTA-sensitized immune cells by pharmacologic activation using bryostatin1/ionomycin (B/I) and common γ-chain cytokines (IL-2, IL-7, IL-15) in order to generate a more effective phenotype of T cells and NK/NKT cells that function together to overcome immune suppression. Herein we report, utilizing qRT-PCR, that breast cancer patients who have remained relapse-free after conventional therapies displayed significant expression in 9/10 CTA transcripts examined in the tumor, whereas those who relapsed with metastatic breast cancer showed no CTA expression; such data demonstrate the rational in epigenetic modulation to induce CTA expression as a therapeutic approach. Further, we demonstrate that upon treatment of human and murine mammary tumor cells with 3 μM Dec for 3 days in vitro, 8/10 and 4/5 CTA transcripts were induced, respectively. We also show that tumor-bearing FVBN202 mice that received injections of Dec also demonstrated induction of CTA expression (5/5) in the tumor. Importantly, upon re-programming CTA-sensitized immune cells harvested from these animals, we observed more robust tumor-specific T cell responses, as demonstrated by a 2-fold increase in the production of IFN-γ. Adoptive cellular therapy (ACT) using ex vivo re-programmed CTA-reactive T cells resulted in significant inhibition of established mammary carcinoma in FVBN202 transgenic mice that had been pre-conditioned with Dec injections. These data, therefore, suggest that traditional barriers in the anti-tumor efficacy of immunotherapy against advanced carcinoma can be overcome by re-programming the crosstalk between tumor and immune cells.
Key Words: Breast cancer, Adoptive immunotherapy, MDSC.

MRNA Mediated T Cell Reprogramming for Adoptive Immunotherapy

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mRNA transfection results in essentially rapid and uniform gene expression in cells and permits simultaneous co-expression of several transgenes. We developed an approach for T cell reprogramming with mRNA coding for chimeric receptors against hematological and solid malignancies.
Autologous T cells can be isolated from peripheral blood of the patient, quickly activated ex vivo and reprogrammed with mRNAs against the tumors. This approach does not dependent on T cell cloning and can be applied to the entire population of T cells.
The efficiency of the method was demonstrated in vivo in Xenogeneic murine models against B cell lymphoma and melanoma tumors.
The introduction of additional chemokine mRNAs can be useful in modulation of lymphocyte activity. We demonstrated in vitro that mRNA coding IL2 can dramatically increase T cell viability in IL2 deficient medium.
Modification of mRNA to increase the duration of its expression in cytoplasm can further improve the applicability of the method. Scaling up the protocols for mRNA synthesis and lymphocytes transfection will make this approach applicable for clinical use. This will make it possible to: 1) use combinatorial lymphocyte programming to increase the efficiency of blood malignancy targeting; 2) eliminate the risk of graft versus host disease that is a possibility with allogeneic lymphocytes; 3) bypass regulatory constraints for DNA transduction, and facilitate transition to clinical trials.
Key Words: Adoptive immunotherapy, Lymphoma, Melanoma.

Cars for Childhood Cancer: Development and Comparison of Permanently And Transiently-Modified T-Cells Targeting All and Neuroblastoma

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Acute lymphocytic leukemia (ALL) and neuroblastoma (NB) account for ~40% of pediatric cancer deaths. While there have been remarkable advances in treatment, the last decade has seen a plateau in survival, suggesting that novel approaches are needed. We have previously demonstrated great clinical success using CD19-directed CAR T-cells (CART19) in adults with CLL. In this study we compare permanently (lenti) and transiently-modified (RNA) CART19 cells in a xenograft model of ALL, as well as the development and comparison of CAR T-cells directed against the NB antigen GD2.
To follow disease progression in vivo, we made the CD19 + ALL cell line Nalm-6 and the GD2 + NB cell line SY5Y bioluminescent. We used our established xenograft model of ALL, and developed xenograft models of NB. All cell lines and T-cells were human in origin.
Mice with established Nalm-6 were given either 1 dose of lenti or 3 doses of RNA CART19 cells, with lymphodepleting Cytoxan (CTX) between doses. Mice treated with lenti T-cells quickly cleared their ALL and remained disease-free. Mice given RNA T-cells showed disease reduction/control and a significant survival benefit, including long-term disease control in some animals.
To test GD2-directed CAR T-cells, NB cells were injected SQ and given 15 days to establish disease, followed by 3 doses of RNA GD2 CAR T-cells with intervening CTX. Intratumoral injection of cells resulted extensive tumor necrosis within 5 days of the first treatment, and significant reduction in tumor volume. We then developed a disseminated model of NB, modeling the clinical circumstances in which adoptive therapy would be used. NB cells were injected IV, reproducibly resulting in liver and bone marrow disease, both relevant metastatic sites. Lenti GD2 CAR T-cells eradicated disease and prevented recurrence long-term. RNA GD2 CAR T-cells significantly slowed the progression of disease, and demonstrated massive expansion within tumor sites.
These data demonstrate the development of transiently-modified CAR T-cells to treat pediatric cancers, highlighting the importance

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of lymphodepletion and optimized dosing schedules. Our pilot NB data suggest that CAR therapy can mediate successful anti-tumor responses in both flank and disseminated models of NB. For antigens such as GD2, use of these transiently-modified T-cells may provide a greater degree of safety than permanently-modified cells, given the very high degree of clinical activity we have observed using CAR-modified cells.

Key Words: Humanized mouse model, Adoptive therapy, Chimeric receptors.

Adoptive T Cell Therapy With a TCR Engineered for Nanomolar Affinity Shows Improved Anti-Tumor Efficacy Compared to the Micromolar Wildtype TCR

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Adoptive transfer of T cell receptor-transduced T cells has shown promise in clinical trials for cancer treatment. Recruitment of CD4+ helper T cells to the tumor could be beneficial, as CD4+ cells play a pivotal role in cytokine secretion as well as promoting the survival, proliferation and effector functions of tumor-specific CD8+ cytotoxic T lymphocytes. However, the TCR affinity that is optimal for redirection of CD4+ T cell activity is not known. Here we show that CD4+ T cells expressing a high-affinity TCR (nanomolar Kd value) against a class I-restricted tumor antigen mediated more effective tumor treatment against an established melanoma tumor model than the wild-type affinity TCR (micromolar Kd value). CD8+ T cells transduced to express nanomolar affinity TCRs were deleted in vivo, but CD4+ T cells with the same TCR resulted in enhanced survival and long-term persistence of effector memory T cells in a subcutaneous melanoma tumor model. The same approach is being examined for efficacy in the treatment of pulmonary metastatic melanoma. Using the same receptor, we have explored strategies to overcome the deletion of CD8+ T cells and mispairing with endogenous TCR chains. Toward these goals, we investigated a chimeric antigen receptor (CAR) that contained, by analogy with scFv-containing CARs, a Vα/Vβ single-chain TCR (scTv) of the high-affinity TCR linked to signaling domains. Unlike nanomolar affinity full length TCRs, nanomolar affinity scTv-CARs introduced into CD8+ T cells were not deleted in vivo, and there was no evidence of pairing with endogenous chains. Furthermore, both the CD8+ and CD4+ T cell subsets transduced with the scTv-CAR were capable of mediating tumor growth control. Overall, our results suggest that TCRs with nanomolar affinity could be advantageous in an adoptive T cell setting.

Key Words: TCR, T cells, Adoptive immunotherapy.

Dual-specific T Cells: Combining Car-engineered T Cells With Oncolytic Virotherapy

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Engineering T cells with chimeric antigen receptors (CARs) to direct them against tumor cells is emerging as a viable option for adoptive T cell therapy. The effectiveness of adoptive T cell transfer is limited by the efficiency of T cell engraftment, degree of tumor infiltration and the immunosuppressive nature of the tumor microenvironment. Oncolytic viruses (OVs) display important biological properties that directly address those limiting factors. Furthermore, OVs can serve as remarkably effective booster vaccines. In an effort to capitalize on the robust boosting properties of the OVs, we have combined OV therapy with CAR-engineered, OV-specific T cells which express a TCR that is specific for the OV and CAR that is specific for the tumor. In mouse tumor models, our experiments have revealed that infusion of vesiular stomatitis virus (VSV), a prototypic OV, following adoptive transfer into tumor-bearing hosts can dramatically boost the CAR-T cells, both CD8+ and CD4+, leading to a marked increase in tumor infiltration. The VSV boost also dramatically affected the distribution of the CAR-T cells. In the absence of the VSV boost, T cells were largely localized to the lymphoid tissues and the tumors. Following the VSV boost, the T cells could be found throughout the body. We have confirmed that the boosting effect requires cognate interaction between the CAR-T cells and the OV-derived antigens. Our current experiments seek to examine the relative benefits of boosting CAR-T cells with with either rhabovirus- or vaccinia virus-based OVs. We are also employing non-oncolytic variants of VSV to determine whether the oncolytic properties of the boosting agent provide a therapeutic advantage over traditional, non-oncolytic booster vaccines. Given the promising clinical outcomes with both these treatment platforms (CAR-T and OV), we believe that our combination approach provides a clinically feasible strategy to maximize the therapeutic potential of these two modalities.

Key Words: Cancer immunotherapy, Adoptive immunotherapy, Chimeric receptors.

COMBINING IMMUNOTHERAPY AND OTHER THERAPIES

Pharmacokinetics and Immunological Effects of Human IL-15/IL-15RA Heterodimeric Complexes in Mice and Macaques

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IL-15 is a member of the γ-chain family of cytokines with non-redundant effects on the immune system, including stimulation of the proliferation, survival and function of NK and T cells. Due to its properties, IL-15 has potential for use in different clinical settings such as cancer and infectious disease immunotherapy. We have previously showed that co-expression of IL-15 and the IL-15 Receptor alpha (IL-15Rα) in the same cell allows for efficient production and secretion of bioactive IL-15 heterodimer in vivo, whereas the single-chain IL-15 is unstable and poorly secreted. In addition, analysis of sera from lymphoablated melanoma patients revealed that circulating IL-15 exists exclusively in association with soluble IL-15Rα (sIL-15Rα), suggesting that the IL-15 heterodimer is the natural biologically relevant form of the cytokine in vivo.

We developed IL-15/sIL-15Rα expression vectors producing high levels of bioactive cytokine. We have also developed stable cell lines overproducing IL-15/sIL-15Rα heterodimers. Delivery in mice and macaques of IL-15/sIL-15Rα purified protein complexes and injection of IL-15/sIL-15Rα-expressing DNA gave similar results, resulting in a great expansion of NK and T cells. We compared pharmacokinetics and biological effects of purified IL-15 heterodimers with E.Coli produced single-chain IL-15. Upon intraperitoneal injection in mice, IL-15 heterodimers showed a favorable pharmacokinetics and induced a greater proliferation of NK and CD8 T cells in spleen and lung, in comparison to single-chain IL-15. Upon intravenous injection in macaques, the plasma half-life of IL-15 heterodimers was 6x longer than single-chain IL-15. Interestingly, subcutaneous injection (s.c.) of IL-15 heterodimers in macaques resulted in persistent bioactive level of plasma IL-15 for up to 72 hours. Five repeated administrations of IL-15 heterodimers in macaques by s.c. route every 3 days resulted in a massive expansion of NK, γδ and CD8 T cells in the peripheral blood.

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In conclusion, in comparison to single-chain IL-15, heterodimeric IL-15 cytokine is more stable in vitro and in vivo, has longer plasma half-life and is more bioactive in mice and macaques. The favorable pharmacokinetics/pharmacodynamics profile of the heterodimers allow lower dose, simple s.c. delivery and lower the possibility of toxicity due to cytokine spike, therefore it is the most favorable form for clinical applications of IL-15.

Key Words: Cancer immunotherapy, Interleukin-15, CD8 + T cells.

In vivo Administration of Interleukin 15 (IL15) Does not Augment Transfer of CD8 + T Effector Cells in Non-human Primates (NHP)

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The adoptive transfer of antigen-specific effector T cells (T_E) is a promising treatment for human malignancies and infections. Persistence of transferred T cells has been identified as a prerequisite for efficacy, but reliably achieving a high magnitude and durable T cell response has proven challenging. Altering the host by administering chemotherapy to induce lymphodepletion improves the survival of transferred T cells in part by removing sinks for homeostatic cytokines such as IL15 and IL7, but has toxicity. Our prior work in a macaque model of T cell transfer showed that deriving CD8 + T_E from CD62L + central memory cells ($T_{CM/E}$) for adoptive transfer provided T cells that when administered to lymphoreplete animals, migrated to memory niches, reverted to long-lived memory T (T_M) cells, and responded to antigen. IL15 is a γ_c-cytokine that maintains T_M survival, and the survival and proliferation of $T_{CM/E}$ cells is enhanced by IL15 in vitro. This motivated us to determine if administering IL15 could further augment the persistence of transferred $T_{CM/E}$ cells in NHP.

Methods: We identified an intermittent 3-week regimen of subcutaneous (s.c.) IL15 (10 μg/kg) that safely increased endogenous CD8 + T_M without boosting CD4 + FoxP3 + regulatory cells. We then treated 7 lymphoreplete macaques with sequential infusions of clonal or polyclonal CD8 + $T_{CM/E}$ (5×10^8/kg) ± IL15. Gene-marking of the $T_{CM/E}$ cells with CD19 or CD20 was used to track migration, persistence, phenotype, and expression of Ki-67 by flow cytometry and/or qPCR after transfer. Retroviral integration was assessed using massively paralleled pyrosequencing.

Results: Intermittent s.c. IL15 yielded plasma levels of > 0.5 ng/mL and enhanced expression of Ki-67 by endogenous and transferred T cells. Despite enhancing proliferation of the infused T cells in vivo, the dose of IL15 used failed to consistently augment the peak level or magnitude of the transferred T cell response compared to that achieved without IL15. Transferred Ki-67^{high} T cells displayed increased signatures of apoptosis in vivo suggesting IL15-induced cycling was balanced by cell death. IL15 did not interfere with the migration of transferred T cells to lymph nodes or bone marrow, or with their formation of long-lived (> 4 y) T_M subsets, including a CD122^{high} T_M subset. Persisting transferred T cells contained diverse retroviral integration sites. The results demonstrate that increased T cell division induced by IL15 is linked to apoptosis, thus limiting the ability to augment a transferred T cell response by administering this cytokine to lymphoreplete hosts. It is possible that higher or sustained doses of IL15 alone or with antigen challenge would improve T cell transfer.

Key Words: T cells, Interleukin-15, Immunotherapy.

Tumor Irradiation Enhances Homing of Vaccine Induced Tumor-Specific CTLS

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The recombinant Semliki Forest virus (rSFV) encoding human papilloma virus (HPV)-E6,7 tumor antigens induces both strong, long-lasting CTL responses in a mouse model of cervical carcinoma and effective eradication of established tumors of HPV-transformed cells. Current therapeutic approaches of cervical cancer patients with advanced disease include radiotherapy combined with chemotherapy. Using a large tissue micro array consisting out of primary tumor tissue of 375 patients with advanced disease we studied the role of tumor infiltrating lymphocytes. Target specific therapies, like immunotherapy, might optimize treatment outcome of these patients. To achieve optimal benefit from immunization protocols, strategies need to be developed that support optimal migration and activity of cytotoxic T lymphocytes (CTLs) into the tumor microenvironment.

We developed a method allowing us to assess the trafficking of tumor-induced, adoptively transferred as well as vaccine-induced antigen-specific CTLs into tumors. To obtain antigen-specific CTLs, donor mice were immunized with (rSFV) encoding HPV-E6,7 tumor antigens. In vitro restimulated E6,7-specific CTLs were CFSE-labeled and adoptively transferred into TC-1 (HPV-transfected) tumor-bearing recipient mice four days after local tumor irradiation. Homing of both tumor-induced effector T cells and adoptively transferred E6,7-specific CTLs to TC-1 tumors was analyzed one day after transfer. Local tumor irradiation induced a significant increase in intratumoral levels of adoptively transferred E6,7-specific CTLs. A similar effect was observed in the infiltration of tumor-induced effector T cells and MDSCs (myeloid derived suppressor cells), when compared with non-irradiated tumors. To assess the effect of irradiation on tumor trafficking of vaccine-induced antigen-specific CTLs, TC-1 tumor bearing mice were locally irradiated and one day later intramuscularly vaccinated. Local tumor irradiation caused a drastic increase in intratumoral levels of both tumor- and vaccine-induced specific CTLs. Intratumoral levels of vaccine-induced specific CTLs were 5-fold higher than intratumoral levels of adoptively transferred E6,7-specific CTLs.

In summary, we demonstrated that vaccine-induced CTLs home into tumors and that local tumor irradiation increases the tumor-homing efficacy of antigen-specific CTLs. This study indicates that rSFV-based immunotherapy combined with tumor radiotherapy could improve treatment outcome in cervical cancer patients with advanced disease.

Key Words: Animal model, Combination immunotherapy, HPV.

Combining the Recombinant Immunotoxin SS1P With the BH3-MIMETIC ABT-737 Induces Cell Death in Pancreatic Cancer Cells

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Background: Our laboratory focuses on the development of recombinant immunotoxins (RITs) for cancer treatment. RITs are antibody-toxin fusion proteins composed of an antigen-binding Fv fused to a 38-kDa portion of Pseudomonas exotoxin A (PE38). SS1P is a mesothelin-targeting RIT that currently is in clinical trials for malignant mesothelioma. In normal tissues, mesothelin is only expressed in mesothelial cells llining the pleura, peritoneum, and pericardium. However, in several malignancies, including mesothelioma and pancreatic ductal adenocarcinoma (PDA), mesothelin is overexpressed. PDA responds poorly to most chemotherapeutic agents, and alternative treatments are clearly needed. We previously reported that, in vitro, mesothelin-expressing PDA cell lines were resistant to SS1P. Despite the inhibition of protein synthesis, cells managed to escape apoptosis due to an aberration in the intrinsic apoptotic pathway. This finding fuelled current efforts to combine our immunotherapy with drugs that can tip the balance in favor of cell death. ABT-737, a BH3-mimetic, binds with high affinity to Bcl-2, Bcl-xl and Blc-w, but not to Mcl-1. High expression of Mcl-1 therefore is a source of resistance to ABT-737. Since

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RITs inhibit protein synthesis, thereby downregulating Mcl-1, we examined whether combinations of SS1P and ABT-737 could induce cell death in PDA.
Methods: Apoptosis of several mesothelin-expressing PDA cell lines was evaluated with FACS (7-AAD and PE Annexin V staining). Protein synthesis inhibition was quantified via 3H-leucine incorporation. Protein levels were evaluated with Western Blotting. SS1P was manufactured at Advanced BioScience Laboratories, Inc. (Kensington, MD).
Results: Treatment with various concentrations of SS1P or ABT-737 separately for up to 72 hours did not induce substantial apoptosis. Combining both compounds, however, led to a significant increase in cell death, either additive or synergetic depending on the cell line, in as little as 24 hours. 3H-leucine incorporation data showed that combining SS1P with ABT-737 significantly enhanced protein synthesis inhibition. This was also obvious by the enhanced speed at which ABT-737 and SS1P downregulated Mcl-1.
Conclusion: Combining SS1P with ABT-737 induces cell death in vitro in several PDA cell lines. However, the different responses to this combination require additional research. Our in vitro findings will be further evaluated in vivo using a recently established xenograft model.
Key Words: Cancer immunotherapy, Apoptosis, Targeted therapeutics.

Selective BRAF Inhibition Impairs Reversible Til Infiltration in BRAFV600E/PTEN-/- Mouse Model of Human Melanoma

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Purpose of the Study: The targeting of the BRAFV600E mutation in human melanoma by small molecule treatment has revealed high response rates, but at short duration. In contrast, immunologically-based approaches, and in particular the blockade of co-inhibitory molecules CTLA-4 or PD-1/PD-L1, induce long-term responses, although overall response rates are relatively low. Consolidation of high response rates may potentially be expected by combining both approaches, in which small molecule treatment-induced antigen release would enhance the efficacy of T cell checkpoint blockade by mAb. Importantly, the optimal timing and drug combinations for such dual treatment have not been established.
Methods/summarized description of the project: To rapidly identify the most promising combinations/treatment schedules for clinical testing we have: 1) crossed Tyr::CreERT2 with conditional BrafV600E and PTEN mutant mice. These mice develop melanomas with high penetrance that can be treated by targeting the BRAF pathway; 2) backcrossed these mice onto the C57BL/6 background, allowing the evaluation of therapy-induced T cell responses; 3) set up the analysis of tumor-infiltrating lymphocytes (TIL) within this model to accurately quantify therapy-induced changes in local immune function.
Results: We found that selective BRAF inhibition impaired TIL infiltration into the tumor environment. This TIL impairment is target-specific as selective BRAF inhibition in BRAFwt tumors did not alter TIL infiltration. Synchronous T cell checkpoint blockade by using CTLA-4 or PD-L1 blocking antibodies in addition to the selective BRAF inhibition could not restore TIL tumor infiltration. Stopping BRAF inhibition restored TIL tumor infiltration within days.
Conclusion: Selective BRAF inhibition and T cell checkpoint blockade might not synergize when applied synchronously, but sequential application might be more promising. Confirmation in human melanoma patients, identification of subpopulations well benefiting, and definition of promising therapy sequence are warranted for clinical implementation.
Key Words: Immunomodulation, Combination immunotherapy, Tumor infiltration lymphocytes.

Ex-vivo Detectable Mage-specific T Cells Correlate With Complete Remission in Metastatic Breast Cancer Patients

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Background: Studies suggest that conventional cancer therapies can, when given sequentially after immunotherapy (IT), boost antitumor immunity. Trials of cancer vaccines followed by chemo- or endocrine therapy have demonstrated a high response rate in metastatic small cell lung cancer and prolonged progression-free survival in prostate cancer. However, immunological correlates were not evaluated during the sequential therapy. Here we report two cases of metastatic breast cancer with an unusual clinical course after IT and present the unexpected immunomonitoring results on sequential treatment.
Methods: PBMCs were tested by ex vivo intracellular cytokine staining for specificity to select tumor antigens (TAA) using overlapping peptides of MAGE-A3, PRAME and control antigens (Proimmune).
Results: Two women with stage IV hormone-receptor positive breast cancer at presentation, large primary tumors with skin involvement and visceral metastases were treated with imiquimod (IMQ) in a clinical trial (Adams et al, Clin Can Res, 2012). IMQ, a Toll-like receptor 7 agonist, was topically applied to cutaneous areas involved by tumor for 8 weeks. While their tumors did not show regression in the study, both women entered a complete clinical response (CR) on the next line regimen with the anti-estrogen fulvestrant several months later. As a CR to fulvestrant is unusual (1% in phase 3 trial), and both CRs are ongoing, we analyzed the patients' PBMCs 2 years after enrollment into the IMQ trial. Both patients had ex vivo detectable Mage-A3-specific T cells (IFNγ +, TNF +, IL4-). Parallel evaluation of pre- and post-IMQ PBMCs of one patient revealed that while not detectable at baseline, IMQ induced Mage-A3-specific T-cells, which were significantly expanded during subsequent endocrine therapy.
Conclusion: Our immune evaluation of long-term disease-free breast cancer patients previously treated with IMQ shows evidence suggestive of in situ vaccination achieved by application of the TLR7- agonist directly onto tumors. Furthermore, we demonstrate that IT-induced antigen-specific T cells can be expanded by subsequent endocrine therapy and persist > 1.5 years. In addition, our data suggest that the induction of ex vivo detectable TAA-specific T cells is a benchmark for true clinical benefit.
Key Words: Topical immunostimulation, Cancer immunotherapy, Combination immunotherapy.

Imiquimod Induced Immune Response

	Date	CEF	MAGE	PRAME
Patient 1 (CR since 9/2010)				
Pre-IMQ	4/2010	1.849*	0	0
Post-IMQ	6/2010	1.980*	0.046	0.030*
On fulvestrant	4/2012	1.293*	0.245	0.051*
Patient 2 (CR since 7/2011)				
Pre-IMQ	3/2010	n.e.	n.e.	n.e.
Post-IMQ	5/2010	n.e.	n.e.	n.e.
On fulvestrant	4/2012	0	0.106**	n.e.

Antigen-specific CD8 + *, CD4 + **T-cells (%), n.e. not evaluable (no functional recovery of PBMC after thawing or limited PBMC numbers), CEF: CMV, EBV, Flu.

PD-1/PD-L1 Blockade After Transient Lymphodepletion to Treat Myeloma

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Early phase clinical trials targeting the programmed death receptor-1/ligand-1 (PD-1/PD-L1) pathway to overcome tumor-

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mediated immunosuppression have reported promising results for a variety of cancers. This pathway appears to play an important role in the failure of immune reactivity to malignant plasma cells in multiple myeloma patients, as the tumor cells express relatively high levels of PD-L1 and T cells show increased PD-1 expression. We have found that T cells in the tissues where myeloma cells reside upregulate PD-1 and are suppressed when this receptor is engaged by PD-L1. In the current study, we demonstrate that PD-1/PD-L1 blockade with a PD-L1-specific antibody elicits rejection of a murine myeloma when combined with lymphodepleting irradiation. This particular combined approach has not previously been shown to be efficacious in other tumor models, and efficacy has only been observed when other immune therapies have been added to this combination. The anti-tumor effect of lymphodepletion/anti-PD-L1 therapy was most robust when tumor antigen-experienced T cells were present either through cell transfer or survival after non-myeloablative irradiation. In vivo depletion of CD4 or CD8 T cells, but not NK cells, completely eliminated anti-tumor efficacy of the lymphodepletion plus anti-PD-L1 therapy, indicating that both T cell subsets are necessary for tumor cell rejection. Elimination of myeloma by T cells occurs relatively quickly as tumor cells in the bone marrow were nearly non-detectable by five days after the first anti-PD-L1 treatment, suggesting that anti-myeloma reactivity is primarily mediated by pre-activated T cells and not newly generated myeloma-reactive T cells. The CD4 T cell requirement is interesting since we speculate that pre-activated CD8 CTL are primarily responsible for the elimination of tumor cells; thus, an ongoing interaction between CD4 and CD8 T cells may be necessary to facilitate tumor cell killing. Anti-PD-L1 plus lymphodepletion did not improve survival in two solid tumor models, suggesting that the effect may be limited to hematologic malignancies. In summary, our results support the clinical testing of lymphodepletion and PD-1/PD-L1 blockade as a novel therapeutic approach for improving the survival of patients with multiple myeloma.

Key Words: Immune-mediated tumor rejection, Multiple myeloma, PD-1.

Sub-lethal Irradiation of Human Colorectal Carcinoma Cells Imparts Enhanced and Sustained Expression of Important Modulators of Effector Ctl Activity

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Sub-lethal doses of radiation can alter the phenotype of target tissue by modulating gene products that may make tumor cells more susceptible to T-cell-mediated immune attack. Previously, we demonstrated that colorectal cancer lines responded to radiation by up-regulating surface expression of CTL relevant proteins including numerous death receptors, cell adhesion molecules and tumor-associated antigens. The present study was designed to determine the extent of CTL relevant changes induced by radiation in human carcinoma cells. Here, several tumor cell lines (SW620, HCT116, Caco-2, Colo205 and WiDr) were examined for their response to various sub-lethal doses of radiation (0-15 Gy). Experiments quantified changes in the expression of genes that could result in enhanced effector CTL activity against irradiated tumor cells. One to seven d post-irradiation, changes in expression of effector costimulatory molecules (OX40L and 41BBL), as well as expression of other molecules involved in effector T-cell activity against target cells (ICOSL and CD70) was examined. All cell lines altered expression of one or more of these molecules post-irradiation. Increased expression could be observed as long as 7-days post-irradiation. In some tumor cell lines, altered expression of these gene products correlated with enhanced killing of irradiated tumor cells by both CEA-specific and MUC-specific CTLs in an in vitro cytotoxicity assay. This was observable as early as 24 hour post-IR and as late as 5 days post-IR. Furthermore we saw enhanced survival of CTLs exposed to irradiated tumor cells detected by decreased numbers of Annexin-V and/or 7AAD positive T-cells. Overall, the results of this study suggest that non-lethal doses of radiation can be used to make human tumors more amenable to immune system attack even in the absence of innate immune response to "danger" from dying cells.

Key Words: Colorectal cancer, Cytotoxic lymphocytes, Ionizing radiation.

Combination Therapy With HSP90 Inhibitors and Interferons Synergistically Increases MHC Expression Leading to Enhanced Tumor Cell Recognition; Implications for immunotherapy

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In an effort to enhance antigen expression on human tumors, making them more susceptible to immune recognition and destruction, we found that Hsp90 inhibitors (iHsp90) can enhance both differentiation antigens and MHC Class I in melanomas and gliomas. An additional series of tumors, including cancers of the breast, cervix, osteosarcoma and lymphomas were treated with a combination of iHsp90 and interferon to assess induction of both Class I and II MHC. Class I MHC was enhanced by iHsp90 in most tumors, but induction of MHC Class II antigens was NOT induced in MHC Class II-negative tumors. Since bothTypes I and II interferon's (IFN) are known to enhance MHC antigen expression, we assessed their ability to synergize with iHsp90s. Importantly, since iHsp90s can inhibit IFN-signaling, we staggered the drug exposure of the tumor cells to the IFNs and iHsp90s to determine if we could circumvent the counteracting effects. While IFN-gamma stimulated strong Class II MHC expression, the simultaneous addition of iHSP90 largely prevented MHC Class II expression. Notably, pretreatment for 24 hours with IFN-gamma allowed iHsp90 to further enhance MHC Class II expression. Similarly, synergistic enhancement of MHC Class I expression was noted with both IFN-beta or IFN-gamma when either was added prior to addition of iHsp90. Staggering treatment with interferon's and iHsp90s yielded strong induction of both Class I and II MHC antigens. In contrast to previous reports showing enhanced T cell recognition of tumors after iHsp90 treatment, the antigens we are assessing are not client proteins of Hsp90, and there is an increase in mRNA levels, cytoplasmic and surface protein expression, and increased induction of gene promoters. While there is an impact on pMEK and pERK expression following iHsp90 treatment, antigen induction is seen on a variety of tumors that vary in their expression of BRAF and NRAS activating mutations. Thus, there is a potential to utilize iHsp90 on a broad array of tumor types and tumor associated antigens that are impacted by Hsp90 expression, although they may not be client proteins of this important chaperon molecule. As both IFN and iHsp90 increased differentiation antigens and MHC, the further increase in MHC expression from the drug combination could be particularly useful for cancer immunotherapy.

Key Words: Immune escape, Combination immunotherapy, Tumor associated antigen.

Effective Activation of Highly Purified NK Cells Expanded ex vivo for the Eradication of Epithelial Ovarian Cancer Cells

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Purpose: Ovarian cancer is the 7th most common malignancy and the 5th most common cause of death from cancers in women.

While many patients initially respond to surgery and chemotherapy, the long-term prognosis is generally unfavorable, with recurrence and development of drug-resistant disease. Therefore, new strategies for ovarian cancers, for examples, immunotherapy or targeted therapy, are needed. For that reason, this study is to evaluated the efficacy and safety of activated NK cells on ovarian cancer cells.
Methods: The patients were diagnosed with endometrioid adenocarcinoma (n = 2), papillary serous adenocarcinoma (n = 2). And two established human ovarian epithelial carcinoma cell lines (SKOV3 and OVCAR3) were used for all experiments. NK cells from Green Cross Corp. (Korea) were obtained and used as effector cells. MTT assay for cell viability, LDH release assay for the cytotoxic activity, and ELISA for the secretion levels of cytokines were measured for the capacity of activated NK cells.
Results: Firstly, an MTT assay was performed to determine the effect of NK cells on the proliferation of cancer cells. NK cells caused the loss of cancer cell viability and proliferation. Secondly, to investigate whether NK cells could have a cytotoxic effect on cancer cells, an LDH assay was performed. SKOV3 showed higher cytotoxicity than OVCAR3 in cancer cell lines and cytotoxicity of papillary serous adenocarcinoma cells was much more pronounced than all other cell types.Thirdly, to analyze the release of cytokines, the levels of IFN-γ, TNF-α and IL-12 were examined. IFN-γ and TNF-α were significantly released at all ratios in all cell types.
Conclusion: Highly purified and activated NK cells can produce various cytokines and many cytokines stimulate NK cell cytotoxicity in vitro. Generally, elevated levels of cytokines are related to cancer, and cytokines appear to play an important role in the progression of ovarian cancers due to their multifunctional roles. Also, highly purified and activated NK cells effectively increased cell cytotoxicity and proliferation, indicating that activated NK cells could effectively eradicate cancer cells. Therefore, highly purified and activated NK cell-based treatment could be an alternative immunotherapy for ovarian cancer patients.
Key Words: Cancer immunotherapy.

Combined Radiotherapy and Immunotherapy Using CPG Oligodeoxynucleotides and Indolamine 2,3 Dioxygenase (IDO) Blockade in Murine 4T-1 Mammary Carcinoma Results in Greater Anti-tumor Effects

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Introduction: In rare instances localized radiotherapy can induce a systemic anti-tumor immune response known as the abscopal effect. Preclinical and clinical data suggest that the immunomodulatory effects of radiotherapy can be enhanced by combining it with immunotherapy. Two clinical trials have demonstrated the effectiveness of combining local tumor irradiation and local intratumoral injection of CpG immunotherapy. This local therapy induced a systemic immune response, as measured by regression of disease at distant, untreated sites, in approximately 27% of patients. There is preclinical data to suggest that the effectiveness of CpG immunotherapy may be limited by induction of the immunosuppressive enzyme, IDO. In this study we examine the effectiveness of combining local radiotherapy and CpG immunotherapy with systemic administration of the IDO inhibitor, 1-Methyl D-Tryptophan (1-MT). We hypothesized that this combination therapy will enhance dendritic cell and T-cell effects on tumor growth and survival.
Methods: Mice with orthotopic 4T-1 tumor were divided into three groups and treated with sham radiotherapy (Group 1), local radiotherapy (Group 2), or local radiotherapy + Immunotherapy (Group 3). Immunotherapy consisted of intratumoral CpG injection and 1-MT administered in the drinking water at 2 mg/mL. Effects of therapy on tumor growth and survival were assessed and flow cytometric analysis was used for immunologic profiling of the tumor, draining lymph nodes, and periphery.
Results: Compared to groups 1 and 2, the combination of radiotherapy and immunotherapy significantly improved overall survival ($P = 0.02$). Likewise, mean tumor growth was decreased in group 3 compared to groups 1 and 2. Flow cytometric analysis revealed increased number of activated dendritic cells in the draining lymph nodes of mice receiving combined therapy. No treatment related toxicities were observed.
Conclusions: Combining radiotherapy and immunotherapy using CpG oligodeoxynucleotides and IDO blockade is superior to radiotherapy alone in the treatment of murine 4T-1 mammary carcinoma and no toxicities were observed with this therapy. Combination therapy increases the activated dendritic cells in tumor draining lymph nodes. Further study is needed to determine the immunologic effects and mechanism of this therapy.
Key Words: Indoleamine 2,3-dioxygenase 1, Abscopal, Radiotherapy.

ADXS11-001 LM-LLO Immunotherapy Targeting HPV-E7: Preliminary Safety and Survival Data From a Phase 2 Study in Indian Women With Recurrent/Refractory Cervical Cancer

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ADXS11-001 (ADXS-HPV) Lm-LLO immunotherapy is a live attenuated Listeria monocytogenes (Lm) bioengineered to secrete a HPV-16-E7 fusion protein targeting HPV transformed cells. The Lm vector serves as its own adjuvant and infects APC where it cross presents, stimulating both MHC class I and II pathways resulting in specific T-cell immunity to tumors. Here we describe preliminary safety and survival data associated with ADXS-HPV treatment in Lm-LLO-E7-015, an ongoing randomized Phase 2 study being conducted in India in 110 patients with recurrent/refractory cervical cancer who have been treated previously with chemotherapy, radiotherapy or both.
Patients are randomized to either 3 doses of ADXS11-001 at 1×10^9 cfu or 4 doses of ADXS11-01 at 1×10^9 cfu with cisplatin chemotherapy. Naprosyn and oral promethazine are given as premedication and a course of ampicillin is given 3 days after infusion thereby clearing any residual vector. Patients receive CT scans at baseline and 3, 6, 9, 12 and 18 months. The primary endpoint is 12 month survival.
As of May 18, 2012, 109 patients have received 255 doses of ADXS11-001The percentage of patients alive at 6 months is 65% (47/72); at 9 months 40% (22/55) and at 12 months 31% (13/42). Although not always observed in immunotherapy that improves survival, tumor responses have been observed in both treatment arms with 4 complete responses (elimination of tumor burden), 5 partial responses ($\geq$ 30% reduction in tumor burden) by RECIST and 33/76 (43%) patients with stable disease ($\leq$20% increase in tumor burden or $\leq$ 30% reduction) in tumor burden. Clinical benefit is not HPV strain specific as tumor responses have been observed in patients infected with several different high risk HPV strains including HPV16, 18, 31, 33, and 45.
ADXS11-001 Treatment has been well tolerated with 95 mild-moderate (Gr 1-2), and 1 Gr3 (dyspnea) adverse events possibly related to the immunotherapy reported in 36% (39/109) of patients. These non-serious adverse events were predominately transient, non-cumulative cytokine release syndrome symptoms that develop within hours of infusion. These symptoms typically responded to symptomatic treatment, or resolved without treatment and were not cumulative or delayed in onset.
ADXS11-001 (ADXS-HPV) immunotherapy can be safely administered to patients with advanced cancer alone and in combination with chemotherapy. ADXS11-001 is well tolerated and presents a predictable and manageable safety profile. Early signs of

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clinical benefit including CRs and PRs in a refractory disease setting merit further investigation. Updated findings will be presented at the meeting.
Key Words: Cancer immunotherapy, Advanced cancer, Active immunotherapy.

Evaluating the Safety of Combined Ipilimumab and Radiotherapy for the Treatment of Metastatic Melanoma

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Background: Ipilimumab (ipi) improves overall survival in patients (pts) with metastatic melanoma (MM) but only a subset benefit. Preclinical and early clinical evidence suggest radiotherapy (RT) may increase the efficacy of immunotherapy. Little is known about the toxicity of administering RT with ipi. We assessed the safety of concomitant palliative RT and ipi and preliminarily examined efficacy.
Methods: After IRB approval, medical records of 17 pts with MM treated with non-brain RT during ipi induction were reviewed. CTCAE v4.0 was used to grade immune-related adverse events (irAEs) occurring after the initiation of RT and $\leq$3 months of the last ipi induction dose. Efficacy was determined by clinician's assessment of improved pt symptoms within the irradiated field and by radiographic assessment of tumors outside of the irradiated field.
Results: From 2005-2011, 17 pts received 19 courses of RT (10 bone metastases, 6 lymph nodes, 1 skin metastasis, 1 perirectal tumor, and 1 periorbital tumor). RT ranged from 20-62.5 Gy total delivered over 1-25 fractions (median 30 Gy in 5 fractions). For 12 pts receiving RT with 3 mg/kg of ipi: 4 had grade (G) 1 irAEs (5 events: 2 diarrhea, 2 rash, 1 hypophysitis); 1 had G2 rash; and 1 had G3 diarrhea. Using a one-tailed Fisher's exact test, the proportion of pts with G3/4 toxicity in this small cohort (1/12; 8.3%) did not exceed the rate of G3/4 irAEs (19/131; 14.5%) in a prior phase III trial of pts receiving ipi at 3 mg/kg ($P = 0.4759$). (Hodi 2010) For 5 pts receiving RT with 10 mg/kg of ipi: 1 had G1 pruritis; 2 had G2 irAEs (rash, cytokine release syndrome); and 2 had G3 irAEs (3 events: 2 hepatitis and 1 rash). Numbers were very small, but the proportion of pts with G3/4 toxicity (2/5; 40%) was higher than the rate of G3/4 irAEs (18/71; 25.4%) in a prior phase II trial of pts treated with ipi at 10 mg/kg,(Wolchok 2009) though no statistical difference was seen ($P = 0.3956$). No pts had G4 events. 3 pts had local inflammation in the RT field (proctitis, cystitis, and localized dermatitis). Within 12 weeks of ipi initiation, 11 pts had improvements within the irradiated field: less pain (5), better vision (1), reduced bleeding (3), and decreased tumor size (2). 3 pts had tumor sites that regressed outside of the irradiated field and 1 pt had overall stable disease. 2 pts died of disease.
Conclusions: This is an early description of the safety of combining RT with ipi induction for MM. RT does not seem to increase the toxicity of ipi at 3 mg/kg. There were likely too few pts treated with RT during ipi induction at 10 mg/kg to assess safety. RT appeared locally effective in palliating sites of MM. Ongoing prospective evaluation is necessary to assess improvements in efficacy compared to ipi alone.
Key Words: Melanoma immunotherapy, Abscopal, CTLA-4.

Personalized Peptide Vaccine in Combination With Chemotherapy for Patients With Advanced Colorectal Cancer

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Background: Despite recent development of various types of chemotherapeutic and molecular targeted agents, the prognosis of advanced colorectal cancer remains poor. Therefore, novel therapeutic approaches, including cancer vaccine, need to be established. We conducted a phase II study of personalized peptide vaccine (PPV) in combination with chemotherapy for advanced colorectal cancer patients.
Methods: Sixty-one advanced colorectal cancer patients (Stage IV, 27; recurrent, 34) who failed at least the first line chemotherapy were enrolled. Median duration of chemotherapy prior to the enrollment was 526 days. A maximum of four HLA-matched vaccine peptides showing higher antigen-specific immune responses were selected based on the titers of IgG against each of 31 different peptide candidates. The selected peptides were subcutaneously administered in combination with chemotherapeutic and/or molecular targeted agents. In order to identify potentially prognostic biomarkers for overall survival (OS), pre-vaccination clinical findings and laboratory data were statistically evaluated.
Results: Median OS from the first vaccination was 355 days with 1-year and 2-year survival rates of 47.5% and 25.0%, respectively. Notably, median OS from the first line chemotherapy was 1127 days with 3-year and 5-year survival rates of 53.6% and 22.8%, respectively. The main toxicity of PPV was skin reactions at injection sites, but no vaccine-related serious adverse events were observed. Among the pre-vaccination factors examined, high levels of inflammatory factors, including IL-6, CRP, and serum amyloid A (SAA), were significantly unfavorable for OS ($P < 0.0001$, $P = 0.0005$, and $P = 0.0001$, respectively). The numbers of previous chemotherapy regimens and serum albumin levels were also associated with OS ($P = 0.0174$ and $P = 0.0444$).
Conclusions: Even in advanced colorectal cancer patients, combined treatment with PPV and chemotherapeutic and/or molecular targeted agents might improve prognosis. To inhibit the inflammatory factors that might negatively affect immune responses to cancer vaccines, a new clinical trial of PPV in combination with anti-IL-6 receptor antibody is currently underway for advanced colorectal cancer patients.
Key Words: Cancer vaccine, Colorectal cancer, Chemotherapy.

Tumor-Directed Myeloid Expansion is Reversed By Radiation Therapy

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The expansion of myeloid cell lineages has been correlated with cancer progression. Myeloid cells are associated with immune suppression, angiogenesis, metastasis, and have been shown to limit the response to therapy. In the MMTV-PyMT spontaneous mammary carcinoma mouse model, we demonstrated that tumor progression was associated with myeloid expansion in the peripheral blood and this expansion correlated with increased numbers of macrophages in the tumor. A number of published studies have demonstrated that tumor-associated myeloid cells suppress T cell responses in vitro and in vivo. Reducing the tumor burden through surgical resection and chemotherapy has been shown to reduce myeloid numbers. However, chemotherapies that reduce tumor burden also have direct effects on myeloid cells, and surgical trauma has been shown to mobilize myeloid cells. We propose that focal radiation therapy of the tumor is an appropriate model to test the hypothesis that myeloid expansion is directly related to tumor burden. To test the consequence of radiation therapy, we treated the 4T1 mammary carcinoma with 3×20 Gy focal radiation in a hind limb model that allows focal radiation of the primary tumor with minimal dosage to radiosensitive regions of the torso. Fresh whole blood was collected from mice over the course of treatment and myeloid numbers were calculated by quantitative flow cytometry. While 4T1 tumor growth caused a dramatic expansion of myeloid cells in peripheral blood, we demonstrate that treatment with focal radiation resulted in a myeloid contraction and tumor control. However, we demonstrate

that residual metastatic disease can prevent myeloid numbers returning to baseline levels observed in tumor-free mice. We also demonstrate that tumor recurrence from residual disease correlated with a return of myeloid expansion and there remained a close correlation between gross tumor weight and myeloid numbers regardless of the route of tumor challenge and the presence or absence of radiation therapy. Interestingly, while T cell numbers did not change with radiation treatment, we demonstrated an increase in CD3 + CD8 + and CD3 + CD4 + FoxP3- proliferation following radiation that correlated with the reduction in myeloid numbers. Our data demonstrates that reduction in tumor burden by radiation therapy causes a significant decrease in an immunosuppressive cell population and a corresponding increase in T cell proliferation. We propose that these data indicates a window of opportunity following radiation therapy for adjuvant immunotherapy.

Key Words: Micrenvironment, Radiotherapy, Macrophages.

Intravesical Treatment of Orthotopic Bladder Cancer With Chitosan/IL-12 Induces Systemic Tumor-Specific Immunity

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Bladder cancer is the sixth most common non-cutaneous cancer diagnosis in the U.S. with an estimated 73,510 new cases and 14,880 deaths in 2012. Although 70-80% of patients are diagnosed early with superficial (non-invasive) disease, bladder cancer has a recurrence rate of approximately 65%. For nearly three decades, Mycobacterium bovis bacillus Calmette-Guerin (BCG) has been the standard of care intravesical immunotherapy for superficial bladder cancer. While touted as the most effective cancer immunotherapy to date, 20-30% of patients who undergo BCG therapy fail initial treatment while 30-50% of BCG responders develop recurrent tumors within 5 years. Furthermore, a significant limitation of intravesical BCG is its inability to generate adaptive tumor-specific immunity leading to the need for long-term maintenance and continuous surveillance of bladder cancer patients. These limitations combine to justify the search for more effective therapies.

Interleukin-12 (IL-12) is under investigation as a candidate to replace BCG. IL-12 has demonstrated remarkable anti-tumor activity against a range of malignancies in preclinical studies. However, schedule-dependent toxicities associated with systemic administrations have limited its clinical use. The intravesical route of administration offers an opportunity to minimize systemic exposure to IL-12 and its associate adverse events. Our previous studies demonstrated that we can enhance intravesical delivery of IL-12 through co-formulation with a solution of chitosan. Chitosan is an abundant, natural polysaccharide derived primarily from the exoskeletons of crustaceans. It is a non-toxic, biodegradable, non-immunogenic unbranched copolymer of glucosamine and N-acetylglucosamine units linked by β(1-4) glycosidic bonds. Three or four weekly intravesical treatments with IL-12 in chitosan solution (chitosan/IL-12) cures 80-100% of mice bearing orthotopic MB49 bladder tumors. BCG, on the other hand, is ineffective in this aggressive model.

Our recent studies demonstrate for the first time that mice cured following intravesical chitosan/IL-12 immunotherapy rejected a distant flank tumor challenge in a tumor-specific manner; 100% of previously cured mice were protected from s.c. MB49 rechallenge, but not s.c. B16 challenge. Protective immunity was found to be dependent on CD4 + and CD8 + subsets as their depletion abrogated protection. These data are the first to demonstrate that an intravesical IL-12-based immunotherapy for bladder cancer can induce a systemic tumor-specific immune response. This is particularly important for patients with highly recurrent bladder cancer as standard-of-care BCG does not generate tumor-specific immunity.

Key Words: Tumor immunity, IL-12, BCG.

Synergistic Therapeutic Effect of Blocking Two Immune Inhibitory Pathways Concurrently in the Context of a Tolerogenic Tumor Microenvironment in vivo

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Over the last two decades, numerous mechanisms have been identified that contribute to immune suppression in the context of a growing tumor. The first therapeutic approach to reach clinical practice is the blockade of CLTA-4 using the anti-CTLA-4 mAb ipilimumab. Despite this therapeutic advance, this regimen is effective in only a minority of patients, and therefore further treatment refinements are required. Based on these notions, we have initiated studies of rational combinations of agents in preclinical models to target two independent inhibitory pathways concurrently, in a logical fashion. For this we intend to uncouple inhibitory pathways largely operational in lymph nodes (CTLA-4) and in the tumor (PDL1-PD1 interactions) combined with treatments that affect T cell anergy (IL-7, LAG-3, 4-1BB), metabolic dysregulation (IDO) or regulatory T cells (Tregs, using CD25-depletion). The first combinations tested have utilized anti-CTLA-4 mAb as an anchor. Using the well-characterized tumor model of B16-SIY cells pre-established in syngeneic mice in vivo, we found a major synergistic effect of combining anti-CTLA-4 with either an IDO inhibitor, with anti-PD-L1 mAb, or with CD25-depletion. The other combinations are being explored similarly. This synergistic therapeutic effect already seen with strategies that have similar agents available for clinical testing points towards logical combination studies to prioritize for clinical trials in cancer patients.

Key Words: Tumor immunity, Adoptive therapy, CTLA-4.

TGFβ IS a Master Regulator of the Pro-immunogenic Effects of Radiotherapy

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Radiation therapy has the potential to convert the tumor into an in situ individualized vaccine by inducing immunogenic cancer cell death and pro-inflammatory cytokines and chemokines; however this potential is rarely realized by irradiation alone. We hypothesized that radiation-induced immunosuppressive factors may hinder its pro-immunogenic effects. Transforming growth factor β (TGFβ) has immunosuppressive function for dendritic cells and T cells and is activated by radiation. Here we tested the hypothesis that inhibiting TGFβ during radiation treatment would induce an immunogenic response.

Poorly immunogenic, highly metastatic 4T1 carcinoma cells were injected s.c. in syngeneic BALB/c mice (day 0). TGFβ neutralizing 1D11 or isotype control 13C4 monoclonal antibodies were given i.p. (200 μg/mouse) every other day from day 12 to 28. Tumors were irradiated with 6 Gy on five consecutive days beginning on day 13. Tumor growth was measured consecutively. Mice were euthanized at day 21 for analysis, at day 28 for enumeration of lung metastases, or followed for survival. Gene expression profiles were obtained using Affymetrix mouse genome 430 2.0 array.

Tumor growth rates and the frequency of lung metastases were similar in mice receiving control antibody or 1D11 alone. Radiation treatment caused significant ($P = 0.0065$) tumor growth delay but did not inhibit lung metastases. In contrast, mice treated with both 1D11 and radiation exhibited significantly greater tumor growth control and reduced lung metastases ($P < 0.0001$), and significantly prolonged survival ($P < 0.005$). As expected, TGFβ signalling was inhibited with 1D11 as measured in CD4 + and CD8 + T cells from tumor-draining lymph nodes at day 21.

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CD8 + T cells producing IFNγ in response to a tumor-specific antigen were detected only in mice treated with 1D11 and radiation. Expression profiles showed that genes associated with immune response and T cell activation were upregulated in irradiated tumors of mice treated with 1D11 compared to other treatment groups. In vivo depletion experiments demonstrated that T cells were essential for the improved tumor control and inhibition of lung metastases of mice treated with 1D11 and radiation.
These data support a critical role for TGFβ as a regulator of the pro-immunogenic effects of local tumor radiotherapy. Inhibition of TGFβ during radiotherapy may promote self-immunization and achieve systemic control of metastatic disease.
Supported by DOD BCRP grant BC100481P2.
Key Words: Breast cancer, Radiotherapy, Immunotherapy.

Intratumoral Chitosan/IL-12 Neoadjuvant to Tumor Resection Reduces Breast Cancer Metastasis

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Interleukin-12 (IL-12) is a potent antitumor cytokine that exhibits significant clinical toxicities after systemic administration. Local delivery strategies capable of maintaining high concentrations of IL-12 in the tumor microenvironment while minimizing systemic exposure are under investigation. We have previously shown that intratumoral injections of IL-12 co-formulated with a solution of chitosan (chitosan/IL-12) can eliminate established primary tumors. Chitosan is an abundant, natural polysaccharide derived primarily from the exoskeletons of crustaceans. Chitosan has been shown to maintain high local concentrations of protein antigens and cytokines through viscous, electrostatic and bioadhesive interactions.
Because of IL-12's well documented ability to generate tumor-specific cell-mediated immunity, we have recently begun to explore the anti-metastatic potential of intratumoral chitosan/IL-12 immunotherapy in a highly metastatic model of breast cancer (4T1). Thus far, we have found that intratumoral chitosan/IL-12 neoadjuvant prior to tumor resection confers a long-term survival benefit (Fig. 1). Specifically, mice bearing flank 4T1 tumors were treated intratumorally with saline, IL-12, chitosan, or chitosan/IL-12 on days 6 and 12 after tumor implantation. All primary tumors were resected on day 15. Mice treated with either saline or chitosan alone died within 33 days after resection. 2/13 mice (~15%) treated with IL-12 alone remain alive more than 100 days after resection. 8/14 mice (~57%) treated with chitosan/IL-12 remain alive more than 100 days after resection. Furthermore, splenocytes from long term survivors demonstrated anti-tumor immunity in a cytotoxic T lymphocyte (CTL) killing assay. Specifically, after 1 week of in vitro stimulation, splenocytes were found to lyse approximately 40% of target tumor cells at an effector:target ratio of 50:1. Based on data obtained thus far, intratumoral chitosan/IL-12 shows promising potential as a neoadjuvant immunotherapy to reduce metastatic disease.
Key Words: Breast cancer, IL-12, metastases.

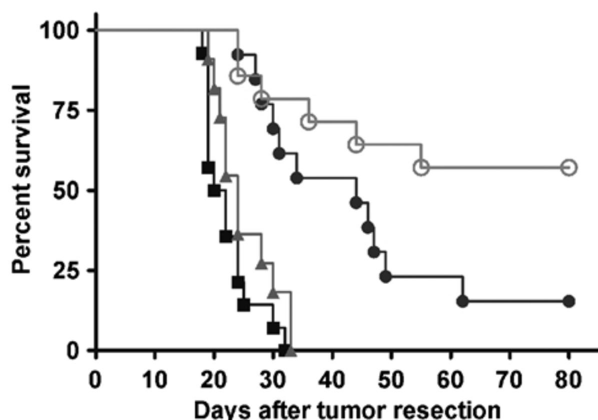

FIGURE 1. Chitosan/IL-12 immunotherapy improves survival following breast tumor resection. Mice bearing 4T1 tumors were given i.t. treatments with saline (filled square), chitosan (filled triangle), IL-12 (filled circle) or chitosan/IL-12 (open circle) prior to resection.

Phase II Trial Daily Pulse Interleukin-2 Therapy During Marrow/Lymphocyte Recovery in Acute Myeloid Leukemia NCT#01289678

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Background: Therapeutic advances in the first line treatment of acute myelogenous leukemia (AML) have yet to step beyond high-dose cytarabine. Allogeneic stem cell transplant (Allo-SCT) can cure patients with relapsed and high-risk AML with a graft versus leukemia immune effect. Growth of clonogenic leukemic blasts can be abrogated following preincubation with lymphokine activated killer (LAK) cells. (Lotzova et al Leuk Res 1987; Lista et al Eur J Haematol 1989) Interleukin-2 (IL-2) will generate LAK cells in peripheral blood and bone marrow. (Foa et al Cancer Res 1991) High-dose IL-2 alone in relapsed AML has achieved complete remissions. (Meloni et al Blood 1994; Marainichi et al Blood 1991) Early absolute lymphocyte recovery after induction chemotherapy predicts a superior survival in AML suggesting a critical role of early immune recovery. (Behl et al Leukemia 2006) Even with an Allo-SCT, slow absolute lymphocyte recovery at day 29 is associated with a higher risk of relapse. (Powles et al Blood 1998) Stimulated LAK cells by IL-2 during this critical early lymphocyte recovery period may enhance a graft versus leukemia immune effect and potentially improve the outcome of first-line AML treatment.
Methods: 9 patients (range ages 33-72) with de novo or secondary AML received IL-2 during induction and/or post-remission treatment marrow nadir recovery. Eligibility criteria included a rising WBC count above 500 mm^3, supported platelet count above 20,000 and hemodynamic stability. IL-2 18 million IU/m^2 was administered over 15-30 minutes daily for 5 days within 7 days of a WBC rising above 500 mm^3.
Results: 3 patients received IL-2 during induction recovery; 3 during re-induction recovery, 3 during consolidation recovery. 1 patient subsequently underwent an Allo-SCT in remission. Median OS 25 months. With a median followup of 36 months (range 12-60 mo), 5 of 9 (55%) treated patients and 4 of 8 (50%) patients without an Allo-SCT, are alive and leukemia free. Of the 5 alive, 3 are over the age of 60 and 2 had complex cytogenetics. Expected toxicities with IL-2 included grade 3 fever/chills (77%) but no respiratory or hemodynamic complications requiring ventilator support.
Conclusions: IL-2 therapy administered during marrow/lymphocyte recovery after standard induction/consolidation chemotherapy in first-line AML treatment achieved a 50% 3-year overall and leukemia free survival without an Allo-SCT.
Key Words: Interleukin-2, Leukemia.

Pseudomonas Aeruginosa Exotoxin T Blocks Apoptotic Compensatory Proliferation Signaling While Inducing Potent Apoptosis in Tumor Cells

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Introduction: Induction of apoptosis has been a major strategy to combat various cancers (Reed, 2006). Resistance to apoptosis,

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however, occurs frequently and contributes to the malignant phenotype (Schattenberg et al, 2011). Although, there may be many reasons for tumor resistance to therapy, one unexplored possibility is that apoptotic cancer cells may promote compensatory proliferation in neighboring unaffected cells. For over two decades, it has been postulated that dying cells induce compensatory proliferation in normal cells as a means to control homeostasis and maintain tissue integrity (Fan et al, 2008a, Fan et al, 2008b, Valentin-Vega et al, 2008, Ryoo et al, 2004). The molecular components involved in the apoptotic compensatory proliferation and the nature of signaling remain unknown. processes.

Results: Recently, we have demonstrated that Pseudomonas aeruginosa ExoT induces potent apoptosis in the cervical adenocarcinoma cells, HeLa (Shafikhani et al, 2008). While investigating ExoT's mechanism of apoptosis, we have identified Crk scaffold protein as a key component of apoptotic compensatory proliferation complex. Our data have uncovered that in apoptotic but still viable cells, this proto-oncogene forms globular complexes within small specialized vesicles which are released from the apoptotic cells and induce proliferation in neighboring tumor cells upon contact (Fig. 2 and Movies 2 & 3 at http://shafikhani-lab.dyndns.org/Videos.html. Allow 30 s for the first movie to upload). Importantly, ExoT induces potent apoptosis but also effectively interferes with the ability of apoptotic tumor cells to promote proliferation in the surrounding cells, thus uncoupling apoptotic programmed cell death from the apoptotic compensatory proliferation signaling. Conclusions: We propose that the induction of apoptotic compensatory proliferation is one of the main mechanisms for reduced efficacy of anti-cancer drugs and enhanced tumor resistance to therapy. We propose that Pseudomonas ExoT because of its unique properties, including its ability to induce potent immunogenic cytotoxicity while blocking apoptotic compensatory proliferation, may be a viable cancer drug that can be used alone or in combination therapy. Understanding the mechanism of compensatory proliferation has a profound therapeutic implication for cancer.

Key Words: Immunogenic cell death, Toxicity, Apoptosis.

Dasatinib Primes Human Cells of Myeloid Origin to TLR-induced IL12 Synthesis

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Dasatinib is a dual bcr/abl/src-kinase inhibitor used for the treatment of bcr/abl + leukemias. Originally it was designed as immunosuppressant and has been shown to inhibit effector T-cell activation. The clinically observed increase of CD8 + T-cells and NK-cells with an LGL-phenotype, associated with colitis and pleuritis, is poorly understood.

We studied the effect of dasatinib on the antigen-specific priming of naïve, human CD8 + T-cells by dendritic cells, using a previously validated priming system (Wölfl et al CII, 2011). This system allows the quantitative and qualitative evaluation of the T-cell response against the melanosomal antigen MART1 after only 10 days of in vitro expansion. Dasatinib was added either at the time of the initial DC/TC interaction, or during maturation of the DC. Furthermore, purified myeloid subsets (dendritic cells, CD14 + monocytes, slan + DC, CD1c + DC) as well as DC from C57/B6-mice and rhesus macaques were evaluated functionally in response to different maturation stimuli in combination with dasatinib.

In striking contrast to the strong suppression observed when dasatinib was present during DC/T-cell interaction, T-cell responses were clearly enhanced, when only the DC had been pre-incubated with dasatinib. Analyzing IL-12p40 production in DC from 10 donors revealed increased responsiveness to LPS (Median% of IL12p40 + DC: LPS/IFNγ: 11.6%, LPS/IFNg/Dasatinib: 24.6%, $P < 0.0001$). Subsequently, higher IL-12 production accounted for T-cells with better TCR avidity leading to increased tumor recognition. This effect was seen in human cells but not in mouse and macaques DC. We identified several src-kinase inhibitors, some of which already in clinical use, with similar effects on IL-12 production in DC. When tested directly ex vivo, the sensitizing effect of dasatinib to TLR-mediated activation was observed in various cell types of the myeloid compartment (monocytes, slan + DC, CD1c + DC). Furthermore, biochemical analysis revealed involvement of src-kinases in the upregulation of IL12-production upon dasatinib treatment, suggesting inhibition of central regulatory pathways of DC activation.

Our findings suggest that increased CD8 + and NK-cell counts observed clinically may be the result of dasatinib-mediated effects on stimulated myeloid cells. Moreover targeting the involved regulatory pathways in DC may greatly improve their function. Such increased functionality of antigen-presenting cells is critical for immunotherapy approaches as well as for the development of a molecularly designed adjuvant.

Key Words: Combination immunotherapy, Dendritic cell, Targeted therapeutics.

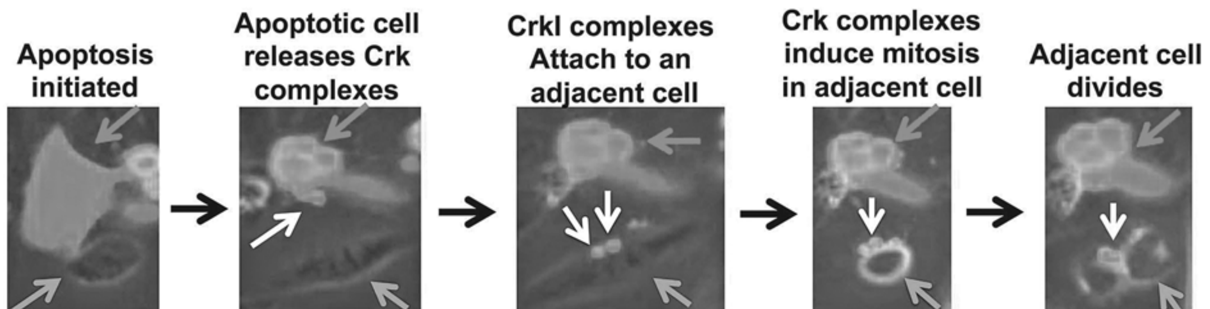

FIGURE 2. Crk is present in apoptotic compensatory signaling vesicles. HeLa cells were transiently transfected with Crk-GFP expression vector. Crk forms complexes (indicated by white arrows) which are packaged in special vesicles that are released from an apoptic cell (indicated by gray arrow) and induced healthy neighbor recipient cells (indicated by white arrow) to divide. Selected Frames from Immunofluroscent timelapse (IF) videomicroscopy (Movie 2) are shown.

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Inhibiting Monocyte/Macrophage-Neuroblastoma Cell Interactions With Lenalidomide Increases Tumor Cell Response to Cyclophosphamide and Topotecan

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Background: New therapeutic strategies based upon understanding the tumor microenvironment may improve the efficacy of chemotherapy and, as a result, long-term survival for patients with high-risk neuroblastoma. We showed that tumor associated monocytes/macrophages (TAMs) and cytokines (IL-6/IL-6R, IL-7, IL-10, IL-8, VEGF, TNFα, and TGFβ1) are prominent in high-risk neuroblastomas. We hypothesize that the milieu created byTAM-neuroblastoma interactions promotes tumor growth and interferes with the efficacy of targeted and non-targeted chemotherapeutic agents. Our specific aims were to 1) characterize TAM-neuroblastoma interactions; and 2) determine if lenalidomide, an immunomodulating drug, inhibits these interactions and improves efficacy of cyclophosphamide-topotecan chemotherapy.

Methods: The effect of TAMs upon tumor cell growth was determined in vitro with co-cultures (Boyden chamber; 15 cell lines tested) and in vivo with subcutaneous co-injection of monocytes and tumor cells into NOD/SCID mice (3 cell lines tested). Using co-cultures, tumor cell DNA synthesis was determined by flow cytometry, pathway activation by Western blotting, and cytokine secretion by Luminex® multiplex assays. The NOD/SCID model was used to determine effects oflenalidomide and cyclophosphamide-topotecan upon growth of luciferase expressing neuroblastoma cell lines.

Results: Co-culture and co-injection into NOD/SCID mice of monocytes with neuroblastoma cells uniformly and markedly enhanced tumor cell growth. Co-culture increased tumor cell DNA synthesis, phosphorylation of STAT3, PI3K (110α, 110γ, p85, p55), AKT, mTOR, S6, 4E-BP1, and IKKβ in both tumor cells and TAMs and secretion of G-CSF, IL-10, IL-1RA, IL-6, IL-7, CCL5, TNFα, and VEGF > 10-fold. Under these same conditions, lenalidomide significantly inhibited tumor cell growth, DNA synthesis, protein phosphorylation, and cytokine secretion. Tumor cells co-injected with monocytes were most effectively eliminated by lenalidomide with cyclophosphamide-topotecan compared to lenalidomide or cyclophosphamide-topotecan alone ($P = 0.01$).

Conclusions: Chemotherapy with cyclophosphamide-topotecan is improved by lenalidomide, which inhibits pro-tumor interactions of TAMS and neuroblastoma cells.

Key Words: Chemokines, Chemotherapy, Immunotherapy.

DC SUBSETS/CANCER VACCINES

Selection of a Control Infectious Disease Vaccine for Cancer Immunotherapy Trials

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An increasing number of clinical trials are being conducted to test vaccines directed against tumor-associated antigens for the treatment of various malignancies. To fulfill the promise of the widespread dissemination of immunotherapies for cancer, it will be critical to employ best practices in the design and conduct of these clinical trials. One significant concern is the ability to induce robust tumor antigen-specific cellular immunity among populations of patients who are older, ill from advanced disease, and potentially lymphopenic as a result of preceding courses of standard-of-care chemotherapy. Thus, the utilization of a vaccine control demonstrating that these patients are capable of responding to immunizing protocols would be beneficial. We have conducted a thorough review of commercially available, licensed infectious disease vaccines in order to identify the most suitable vaccine for this purpose. An ideal control vaccine would have demonstrated ability to induce anamnestic antigen-specific T cell responses that could be consistently measured. Other desirable characteristics would include minimal adverse effects, a relatively low prevalence of natural immunity and/or vaccination rates in an older adult population, and a possibility of clinical benefit, making the vaccine acceptable to all trial enrollees. We eliminated potential vaccines if they were contraindicated for immunocompromised patients, had substantial adverse effects in any population including healthy patients, were not licensed for older adults, were only approved for high risk subjects, were no longer commercially available, induced only T cell-independent responses, or were associated with potentially high levels of pre-existing immunity due to exposure from childhood and/or subsequent booster vaccination. Of the remaining possible vaccine candidates, we suggest that hepatitis A virus vaccine may have the best combination of features including minimal adverse effects, relatively low natural exposure and vaccination rates, and robust cellular immune responses after a single dose that can be readily measured. We will discuss the advantages and disadvantages of this and other potential choices.

Key Words: Cancer vaccine, Immunization, Cellular immunity.

Montanide™ ISA 51VG and Montanide™ ISA 720VG, Adjuvants Dedicated to Human Therapeutic Vaccines

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Montanide™ ISA 51 VG and Montanide™ ISA 720 VG are adjuvants rendering stable water in oil (W/O) emulsions when mixed with antigenic media. Montanide™ ISA 51VG is based on blend of mannide monooleate derivative surfactant and mineral oil, whereas Montanide™ ISA 720 VG uses a non mineral oil. These adjuvant when mixed with antigenic media into W/O emulsion formulations are allowing a sustained release of the antigen and a prolonged stimulation of the immune system. They are strong inducers of danger signal (signal 0) through proinflammatory environment and enhancement of interaction between antigen and dencritic cells (DC). Their way of action, independent of MyD88 and Toll Like Recpetors (TLR), is based on Pattern Recognition Receptor (PRR) activation, as NOD- like receptor, specifically, NOD2 protein, leading to nuclear factor kappa-light-chain-enhancer (NF-κB) translocation. This pro-inflammatory response creates a local immunocompetent environment and triggers DCs recruitment before it helps for maturation and migration of these cells.. This mechanism induces then an important specific antibody and Interferon gamma. TH1 polarized response, and potent response cytotoxic T lymphocytes (CTL). WO emulsified vaccines act as substitute of danger signals, but they provide this information in a safe manner through prolonged half-life of the antigen, enhanced innate cell infiltration into the site of injection, improved antigen presentation by antigen-presenting cells and increased production of immunomodulatory cytokines and chemokines.

Montanide ISA 51 VG and Montanide ISA 720 VG are included in numerous clinical trials up to phase III in the world, and Montanide ISA 51 VG is a component of a therapeutic vaccine against NSCLC (CIMAVax EGF) registered in two countries.

Key Words: Therapeutic vaccine, Cancer vaccine, Adjuvant.

Effect of Age on Immunity and Responses to Breast Cancer Vaccination

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Background: Elderly patients (aged > 65) suffer nearly half of all malignancies in the United States and nearly two thirds of all malignancy-related deaths. By the end of the current decade, most cancers will be diagnosed in elderly patients. Yet the clinical impact

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of immune senescence, which is marked by T cell unresponsiveness, on cancer vaccines remains largely unknown. No published studies have systematically compared cancer vaccines in younger and older patients. As vaccinated patients are typically middle-aged or elderly, we sought to determine if age might impact vaccine efficacy.
Methods: We conducted a meta-analysis of immune monitoring data and clinical outcomes from three clinical studies of breast cancer vaccines conducted by the Tumor Vaccine group at the University of Washington since 2004. Two studies tested a HER2/neu-based peptide vaccine (n = 22 and n = 38, respectively) while a third tested a HER2/neu-based plasmid DNA vaccine in a dose-escalation study (n = 22 in each arm, n = 66 total). These studies enrolled a total of 126 patients with stage III/IV breast cancer, with all patients receiving a tetanus vaccine on the same day as the study vaccine. Immune responses were monitored throughout the studies using the IFNγ ELISPOT assay to measure the frequency of antigen-specific cytokine-secreting cells in circulating PBMC. We correlated maximum immune response with patient age and clinical outcome using Pearson's coefficient of correlation.
Results: The average patient age across the three studies was 50.6 + /− 9.3 years and ranged from 30 to 77 years. For patients with matched ELISPOT results for multiple time points (n = 99), we found detectable baseline responses to tetanus in 80% (n = 79) and boosted responses post-vaccination in 79% (n = 78), indicating that these patients are able to respond to vaccination, but the magnitude of response varied. Age and maximal antigen-specific IFNγ responses to tetanus were inversely correlated ($R = -0.2159$, $P = 0.0301$) across all three studies. In the first peptide vaccine study, which correlated the development of post-vaccination intramolecular epitope-spreading to long-term survival, we found that age was also inversely correlated with epitope-spreading ($P = 0.0527$, n = 20). For 3 out of the 9 HER2/neu epitopes analyzed, maximal antigen-specific post-vaccination responses showed significant ($P < 0.05$) negative correlation with age.
Conclusion: Long-term follow-up and analyses remain in progress. However, these observations indicate that age may impact cancer vaccine efficacy and suggests that mitigation of age-associated immune defects may be important to optimize and potentially enable successful breast cancer vaccines and maximize their clinical impact.
Key Words: Breast cancer, Cancer vaccine, Immunotherapy.

3-DAY Dendritic Cells for Postremission Vaccination in AML: Characterization of TLR-AGONIST Matured DCS Expressing the Leukemia-Associated Antigens WT1 and Prame

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We have designed a new generation of dendritic cells (DCs) optimized for the use in cell-based immunotherapy of cancer patients. Our goal was to tailor these DCs to be used for vaccination in acute myeloid leukemia (AML) patients with a high risk of relapse after intense induction/consolidation therapy in order to eradicate minimal residual disease. A three-day manufacturing protocol was established using a cytokine cocktail containing a synthetic TLR7/8-agonist for generation of monocyte-derived mature DCs with improved immunogenicity. For induction of a specific T cell-based anti-AML response against residual tumor cells, our DCs were loaded with RNA encoding the leukemia-associated antigens WT1 and PRAME.
Additionally, DCs transfected with RNA encoding CMV-pp65 serve as a helper and surrogate antigen in our proposed clinical phase I/II trial. In this study, we present the careful evaluation of our 3d DCs generated from healthy donors and AML patients after consolidation therapy. Following RNA electroporation and cryopreservation, we could ensure a fully functional phenotype of the autologous dendritic cells. Our studies demonstrated high and controlled protein expression of all three antigens following RNA transfection, which was also stably detected after cryopreservation (WT1: 90-93%, PRAME: 35-80%, CMV-pp65: 78-93%, n ≥ 3). Additionally, expression of common DC surface markers was not altered by these processing steps. To ensure functional integrity of our DCs, the ability to secrete the critical cytokine IL12p70 upon T cell encounter - an important characteristic of our TLR matured DCs - was analyzed in a signal-3 assay with CD40 ligand transfected fibroblasts. We observed a high IL12p70 secretion of RNA transfected DCs even after thawing as compared to IL10 (n ≥ 3 for each antigen). We therefore can conclude that electroporation and cryopreservation did not alter this capacity. Furthermore, cryopreserved DCs expressing the different antigens also displayed a high capacity both for reactivation of antigen-specific pre-primed effector cells and for priming of naïve T cells in vitro, showing proper processing and presentation of the introduced antigens. These studies thereby demonstrate that our manufacturing protocol yield improved DCs with a high potential to initiate long-lasting anti-leukemic responses in patients with AML.
Key Words: DC-based vaccine.

Characterization of Two-Day Derived Alpha Dendritic Cells Suitable for Cancer Immunotherapy

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Rational: The exposure of immature dendritic cells (iDCs) to pro-inflammatory cytokines (IFN-α, IFN-γ, Poly I:C, TNF-α, IL-6 and IL-1β) generates in 7 days a Type I polarized Alpha-DC (aDCs) that produce significant levels of IL-12 required for efficient stimulation of anti-tumor CD8 + T-cells. For this reason aDCs are an important alternative for cancer immunotherapy. Similar to what has been described for mature DCs (mDCs) obtained by culturing iDCs in IL-1β, IL-6, TNF-α and PGE2 for 24 hours - named hereinafter Fast-Standard DC (Fast-Std-DCs) - we consider that a reduction in seven to two days in the generation time of aDCs would be advantageous for its clinical use. In this work, we present the phenotypic and functional analysis of aDCs produced after two days in culture (Fast-aDCs).
Methods: Adherent monocytes were induced to iDCs with GM-CSF and IL-4 for 24 hours and mDCs were induced with the respective maturation cocktail for additional 24 hours. DCs phenotype was measured by flow cytometry using CD80, CD83, CCR7, CD123, CD11c, HLA-DR, CD209 and CD14 antibodies. Secreted cytokines were detected in culture supernatant by CBA. CD8 + T-cells from a breast cancer patient and CD4 + T-cells from an individual with latent tuberculosis were stimulated in vitro with fresh or freeze-thawed Fast-Std-DCs and Fast-Std-DCs mDCs pulsed with HER-2 (369-377) peptide or a pool of M. tuberculosis peptide-antigens respectively. The proficiency of mDCs to stimulate T-cells was assessed by measuring effector and memory T-cells sub-populations that produce IFN-γ and TNF-α upon in vitro stimulation with antigen pulsed mDCs.
Results and Conclusions: In response to maturation, both Fast-Std-DCs and Fast-aDCs up regulate CD80, CD83, HLA-DR and down regulate CD209 and CD14 markers. In contrast, CCR7 was expressed only by Fast-Std-DCs and IL-12 was only secreted by Fast-aDCs. Functional assays showed that both types of peptide pulsed DCs induced the production of IFN-γ and TNF-α by CD4 + T-cells. That similar level of activation was obtained despite the use of fresh versus freeze-thaw peptide pulsed mDCs suggest that after thawing, peptide pulsed mDCs that had been stored frozen maintain intact its capacity to efficiently present antigens and stimulate CD4 + T cells. Finally Fast-aDCs were more efficient than Fast-Std-DCs to stimulate IFN-γ production by HER-2 tetramer specific CD8 + effector cells T-cells. Altogether,

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these results led us to argue that Fast-aDCs obtained from monocytes in two-day protocol lead to mDCs suitable for cancer immunotherapy.
Key Words: Cancer immunotherapy, DC-based vaccine, Dendritic cell.

Transcriptional Signatures in Dendritic Cells: Correlation of Patient Variability With Clinical Outcome

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In previous preclinical studies we have defined factors affecting monocyte derived dendritic cells (DCs) variability and hypothesized inter-patient variability (IPV) as a major affecter of DCs functionality and potency in clinical settings. Therefore, we tested our hypothesis by analyzing gene expression profiles of 74 TARP-antigens pulsed DCs vaccines administered to 14 patients with biochemically relapsed prostate cancer in the context of a phase I clinical trial at the NIH. In particular, we sought to correlate the profiles with clinical outcome (measured as an increase in PSA doubling time) with the intention to identify potential biomarkers of biological activity correlating with clinical effectiveness. First, we tested gene expression profiles for the presence of a shared signature of responsiveness. Even though a signature able to distinguish responders versus non responders was found (*t*-test with *P*-value <0.001, 655 genes differentially expressed), the evaluation of IPV dependent effects suggested that most of the discovered signature might be reflecting IPV and not clinical response. Based on this observation we focused more on IPV and found that genes showing the highest IPV were enriched in inflammatory response, antigen binding and chemotaxis related genes, suggesting the presence of functional differences among DCs generated from different patients. Next, we tried to detect unique features shown by non responders DCs by analyzing the expression level of genes showing the highest IPV. Such an approach revealed that DCs of non responders showed a downregulation in at least one of the following gene ontology families: cell death of immune cells, maturation of DCs, and differentiation of monocytes gene. In conclusion, IPV could be a major factor affecting DCs function and has to be rigorously characterized in order to develop assays for the evaluation of the quality and potency of DCs used in cancer vaccines.
Key Words: DC-based vaccine, Biomarker, Prostate cancer.

Melanoma Cancer Stem Cells of Mesenchymal and Neural Crest Origin Used in Autologous Dendritic Cell-based Immunotherapy Result in 50% 5-year Survival Rates

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The ideal source of antigen in an immunotherapeutic approach to cancer is to use the patient's own tumor products. Bulk preparations lack large amounts of antigen from the most aggressive phenotypes, namely tumor initiating or cancer stem cells. Our latest approach uses specific media formulations to isolate and propagate putative cancer stem cells from patient tumor samples to quantities necessary for loading dendritic cells. Previously, our standard approach using non-cancer stem cell specific media was labor intensive and lengthy with an average production time of 3.8 months (range 0.6 to 22.3 mo, median 3.1). This resulted in delayed time to treatment with only 29% of the patients who submitted a sample receiving therapy. Frequently, over growth of normal fibroblast required extensive manipulation by skilled technicians which made the process expensive. However, characterization of these cell lines by flowcytometry demonstrated the enrichment for cells of mesenchymal and neural crest origin (CD146 and CD271, respectively) which have been described as melanoma stem cell markers. Comparison of these cell lines versus the original bulk enzyme digest samples demonstrated that they were enriched for either CD146 and/or CD271 (78.5 ± 8.3% vs. 26.9 ± 5.8%) after purification and expansion. Examination of 35/42 cell lines used in a randomized phase II clinical trial revealed consistent expression these markers in the purified tumor cell lines (35.2 ± 3.9% CD146 + /CD271-, 41.5 ± 4.3% CD146 + /CD271 + , 16.9 ± 4.0% CD146-/CD271-, and 6.4 ± 1.9% CD146-/CD271 +) however no associations could be made with survival and expression of one or both markers. But using these cells as the antigen source in an autologous dendritic cell therapy resulted in 50% 5-year survival in patients with stage IV melanoma (n = 54). Using excess cryopreserved samples and our proprietary media formulation, we were able to reduce the production time to 2 months and increase the success rate to 80%. This process also resulted in increasing the purity of the cancer stem cells from ~70% to > 90% based on these known cancer stem cell markers. In addition, contaminating fibroblasts were eliminated with minimal skilled manipulations. Lastly, the approach is now being optimized for a more closed and uniform system that may make it feasible for automation and scalability which will reduce the cost of delivering the patient-specific therapy.
Key Words: Melanoma immunotherapy, Flow cytometry, DC-based vaccine.

A Novel Dendritic Cell-Based Vaccine for HER-2-positive Early Breast Cancer

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Twenty-seven patients with HER-2/neu over-expressing ductal carcinoma in situ (DCIS) of the breast were enrolled in a neoadjuvant vaccine trial for safety and immunogenicity of IL-12-secreting dendritic cells pulsed with 6 promiscuous MHC class II-binding peptides plus an additional two HLA-A2.1 class I-binding peptides. The DCs were activated with a combination of interferon-gamma plus a special clinical grade bacterial lipopolysaccharide and injected directly into groin lymph nodes at 4 weekly intervals prior to scheduled surgical resection of DCIS. Post-immunization we observed Th sensitization via ELISPOT against at least one of the 6 promiscuous class II peptides in 22 of 25 (88%) evaluable subjects, while 11 of 13 (84.6%) HLA-A2.1-positive subjects had responses to at least one of the class I peptides. For many of the immunized subjects, these anti-peptide responses proved to be quite durable, with enhanced ELISPOT activity extending beyond 50 months post-vaccination. Interestingly, at the time of surgery, 5 of 27 subjects (18.5%) displayed no evidence of residual DCIS, and among the 22 subjects with remaining DCIS, HER-2 expression fell to virtually undetectable levels in 11 (50%). Immunohistochemical analysis and comparison of pre-immunization biopsy with post-vaccine surgical specimens also showed elaborate infiltrations of CD4 + (Th), CD8 + (CTL) and CD20 + (B cell) lymphocytes into the area of DCIS as an apparent consequence of vaccination. Also of note was a pronounced difference in responsiveness between subjects with estrogen receptor (ER)-expressing disease and those without estrogen receptor. For example, after vaccination no residual DCIS was found in 40% of the ER- subjects, compared to only 5.9% of the ER + subjects. In addition, continued high HER-2 expression was found in only 10% of the ER- subjects, while it remained elevated in 47.1% of the ER + subjects ($P < 0.04$). Finally, within the ER- subject population, we have not observed any in-breast recurrence events, with the earliest patients of this trial now out beyond 88 months post-vaccination. These results indicate that this approach may hold promise for the secondary prevention of early breast cancer, particularly in the ER- patient population.
Key Words: Breast cancer, Cancer vaccine, Dendritic cell.

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A Phase 1 Trial of a Novel Vaccine Targeting NY-ESO-1 to the Dendritic Cell Receptor DEC-205 in Combination With Toll-like Receptor Agonists

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Targeting protein antigens to the DEC-205 receptor on dendritic cells was pioneered by Ralph Steinman and shown to induce potent and broad immunity in preclinical models. CDX-1401 vaccine consists of a human mAb specific for DEC-205 fused to full-length tumor antigen NY-ESO-1. A phase 1 trial assessed the safety, immunogenicity and clinical activity of escalating doses of CDX-1401 plus the toll-like receptor (TLR) agonists resiquimod and/or Poly ICLC. 45 treated patients (pts) had advanced malignancies that had progressed after any available curative/salvage therapies (21 melanoma, 6 ovarian, 5 sarcoma, 4 NSCLC, 4 colorectal, 5 other); 87% had distant metastases at entry; 60% had NY-ESO-1 + tumors as per central analysis of archived tumor; median age = 64 years; 51% were male. 41 completed ≥ 1 treatment cycle (4 CDX-1401 doses plus adjuvant over 6 wk); 10 were retreated [median (range) = 10 (6 to 20) doses]. 13 pts had stable disease [median (range) = 5.6 (2.4 + to 13.4) months], and 4 pts had measurable tumor shrinkage (-2, -8, -20 & -21%). 7 pts completed 2 years on study; 11 remain in follow up. Treatment was well tolerated without dose limiting toxicities; treatment-related toxicities (all $\leq$ Grade 2) included injection site reaction (69%), fatigue (22%), and nausea (9%). Significant anti-NY-ESO-1 titers (up to 1:800,000) occurred in 80% of pts. ~60% of pts with NY-ESO-1 + tumors had significant anti-NY-ESO-1 titers at baseline and most increased after vaccination. Humoral responses were elicited in both NY-ESO-1 + and - pts. NY-ESO-1-specific T cell response (IFN-γ ELISPOT) was absent or low at baseline, but increased post-vaccination in ~50% of pts. Evidence of NY-ESO-1 specific CD4 or CD8 T cell responses were observed using intracellular cytokine secretion and pentamer analysis in selected samples. Similar immune responses were observed with adjuvants, alone or in combination, with no clear dose response in these small cohorts. This is the first study documenting that dendritic cell targeting through DEC-205 can safely lead to robust humoral and cellular immunity when combined with TLR agonists in cancer patients.

Key Words: Therapeutic vaccine, Cancer immunotherapy, DC-based vaccine.

Randomized Trial of two Active-specific Immunotherapy Products Derived From Autologous, Proliferating, Self-Renewing Tumor Cells in Patients With Metastatic Melanoma

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Only 10% of metastatic melanoma patients survive five years, even though many can achieve substantial tumor reduction by surgical resection and/or radiation therapy and/or systemic therapy. An effective, non-toxic, consolidation immunotherapy may benefit such patients. Self-renewing autologous tumor cells may be an ideal source of tumor immunogen for patient-specific active specific immunotherapy because of expression of antigens associated with tumor stem cells and/or progenitor tumor cells, and unique patient-specific neo-antiogens. We initiated a randomized trial to compare two promising patient-specific immunotherapy cell products that were associated with 5-year survival rates of 28% and 50% in successive single arm trials involving 74 and 54 patients respectively. Patients had to have a diagnosis of metastatic melanoma and availability of an autologous melanoma cell line. Patients were stratified by whether their most advanced stage had been regional or distant metastases, and by whether they had measurable disease at the time of treatment, then they were randomized to receive irradiated autologous proliferating tumor cells (TC) or autologous dendritic cells (DC) loaded with antigens from such cells. Both products were injected subcutaneously in 500 microgram of granulocyte-macrophage colony stimulating factor, weekly for three weeks and then monthly for five months. Accrual closed prematurely when the cell biology laboratory was closed by the funding institution for budgetary reasons. An analysis was performed in September 2011, when 21/42 patients were deceased, no patients were lost to follow up, and all surviving patients had been followed for at least 6 months after randomization. Patients in the two arms did not differ in baseline characteristics, and all patients received prescribed therapy. Treatment was well tolerated. Survival was superior in the DC arm (HR 0.27, 95% CI 0.098-0.729) with median survival not reached versus 15.9 months, and 2-year survival rates of 72% versus 31% ($P = 0.007$). An updated survival analysis will be performed at the end of September 2012 by which time additional deaths will have occurred, and all surviving patients will have a minimum of 18 months of follow up.

(Supported by the Hoag Hospital Foundation, NCT00436930).
Key Words: Melanoma immunotherapy, DC-based vaccine, Autologous Vaccine AHICE.

Preoperative Sipuleucel-T Results in Tumor Lymphocyte Infiltration in Prostate Cancer Specimens: Evidence of Immune Activation and Response Within The Prostate Tumor Microenvironment

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Background: Sipuleucel-T is an FDA-approved autologous cellular immunotherapy for patients with asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer (mCRPC). To date, studies of sipuleucel-T have investigated the immune response in the autologous product and in peripheral blood. The immune effects of sipuleucel-T in prostatic cancer tissue are unknown.

Methods: NeoACT (P07-1; NCT00715104) is an open-label, Phase 2 study of patients with localized prostate cancer who received sipuleucel-T (3 infusions at approximately 2-week intervals), beginning 6-7 weeks prior to radical prostatectomy (RP). The primary endpoint was the change in the frequency of infiltrating lymphocytes from pre-treatment (prostatic biopsy material) to post-treatment with sipuleucel-T (RP specimens), as assessed by immunohistochemistry (IHC).

Results: Of 42 enrolled patients, 37 received all 3 pre-RP sipuleucel-T infusions and were evaluable by IHC. Significant increases (> 3 fold) in infiltrating CD3 +, CD4 +, and CD8 + T cells were observed at the tumor rim (where benign and malignant tissues interface), compared with the pre-treatment biopsy (all pairwise $P = 0.0001$). The majority of CD3 + T cells at the tumor rim were PD-1 and Ki-67 positive. CD4 + /FoxP3 + T cells were increased at the tumor rim (~2 fold; $P = 0.004$), but represented a small proportion of the observed T cells. The frequency of CD20 + B cells was also increased at the tumor rim. This type of lymphocyte infiltration was not seen in a cohort of 12 concurrent cases that did not receive neoadjuvant treatment. Systemic B cell and T cell activation was observed following treatment. Increased expression

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of activation markers CD134, CD137, CD278, and CD279 were observed in circulating T cells following the first infusion. Activated mature B cells increased during dose preparation in all 3 doses ($P < 0.01$); memory B cells were progressively increased ($P < 0.05$ third vs. first product). In patients with available peripheral blood samples to assess antibody response (n = 13), significant increases in prostatic acid phosphatase (PAP)-specific IgG and IgM antibodies were observed at the time of RP compared with baseline ($P < 0.05$; Wilcoxon signed rank test).

Conclusions: Systemic administration of sipuleucel-T was associated with an antigen-specific immune response, and resulted in localized T and B cell infiltrates in resected prostate cancers at the interface of the benign and malignant tissue. This is the first report of immune effects of sipuleucel-T in the prostate tumor microenvironment.

Key Words: Cancer immunotherapy, Prostate cancer, Tumor microenvironment.

Phase I Peptide Vaccine With Montanide ISA-51 VG in Children With Refractory Central Nervous System (CNS) Tumors

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Background: CNS tumors are the second most common cancer in children and the leading cause of mortality due to disease. New therapies are desperately needed.

Specific Aims: The primary aim of this phase I study was to determine the safety and feasibility of administering HLA-A2 restricted, tumor associated antigenic peptides with Montanide ISA-51 VG to children with refractory CNS tumors. Secondary aims were to evaluate immune response to the vaccine and tumor response.

Methods: Each vaccine consisted of HLA-A2 restricted peptides targeting epitopes on the tumor associated antigens Her2, Trp2, EphA2 and gp100 mixed with Montanide ISA-51 VG as the immune adjuvant. The neoantigen KLH was given with the first vaccine as a control. Patients received the vaccines divided into 2 subcutaneous injections on weeks 1, 4 and 7. Immune responses induced by the vaccine were evaluated by tetramer and intracellular cytokine staining.

Results: 15 patients, females = 8, median age 12 years (range 7-20 y) were treated between August 2009 and May 2012. Diagnoses included pilocytic astrocytoma = 1, low grade glioneuronal tumor = 1, pilocytic/pilomyxoid tumor = 2, anaplastic astrocytoma = 3, DIPG = 2, radiation-induced glioblastoma = 1, and ependymoma = 3. Two patients had unbiopsied presumed low grade astrocytomas. One patient with an ependymoma was removed after only 2 immunizations because of progressive disease. 14 pts received all 3 vaccines. Several patients had grade 1 local skin reactions at the injection sites. No patients had grade 2 or higher adverse reactions related to the vaccine. Analysis of immune response shows induction of T cell responses to the tumor associated antigens. Impressively, most patients evaluated so far had detectable T cell responses to gp100 and Her-2 post vaccination. Furthermore, both antibody and T cell responses to the control antigen KLH were detected in most patients. Five of 6 patients with low grade astrocytomas have had stable disease for a median of 24 months (range 6-36 mo). Three patients with anaplastic astrocytomas have stable disease for 16, 24, and 24 months.

Conclusions: Vaccine therapy using tumor associated antigenic peptides with Montanide ISA-51 VG was well tolerated. Despite being heavily pre-treated, these children were able to mount both humoral and adaptive immune response. Stable disease was seen in children with refractory low grade and high grade gliomas.

Key Words: Glioblastoma, Cancer vaccine, Active immunotherapy.

Semi-allogeneic Cancer Vaccines

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Experimental results from studies with inbred mice and their syngeneic tumors indicated that the inoculation of semi-allogeneic cell hybrids (derived from the fusion between syngeneic tumor cells and an allogeneic cell line) protects the animal host from a subsequent lethal challenge with unmodified syngeneic tumor cells. This approach appears to increase the immunogenicity of a tumor and is called heterogenization, which can be achieved by fusing patient-derived tumor cells with designated allogeneic cells. The purpose of heterogenization is to force the host immune response to recognize tumor-associated antigens in the context of allogeneic HLA-I or II molecules or in proximity of strong non-self antigens. The allogeneic/non-self antigen would provide a strong co-stimulatory signal to enhance anti-tumor immune responses. We reported on the use of semi-allogeneic vaccines as stimulators of HIV-envelope-specific cytotoxic T lymphocytes (CTL) and we proposed that semi-allogeneic cell hybrids functionally mimic antigen-presenting cells (APC) by concomitantly stimulating alloantigen-specific T helper (Th-1) cells via allogeneic HLA, and antigen-specific CTL precursors via antigen presentation through self-HLA. We also proposed that the Th-1 cytokine response, induced through alloantigen-specific help, activates more efficiently antigen-specific CTL and that the cytokine-rich microenvironment of allograft rejection is crucial to attracting dendritic APC. Using this approach, we were allowed by the Food and Drug Administration to conduct two Phase I studies in patients with disseminated melanoma and metastatic adenocarcinoma. We determined that treatment of cancer patients with irradiated semi-allogeneic vaccines is associated with minimal or no toxicity and can induce a specific anti-tumor immune response, measured by a positive delayed-type hypersensitivity (DTH) to irradiated autologous tumor cells injected intra-dermally. We can generate unlimited amounts of tailor-made semi-allogeneic vaccines for melanoma patients using a single blood draw. This approach can readily be translated into a Phase II randomized clinical trial as an adjunct to standard therapy.

Key Words: Cancer vaccine, Active immunotherapy, Combination immunotherapy.

"Bacteriomimetic" Nanoparticles for Immunotherapy Against Cancer

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Immunotherapy represents a potential and innovative means to combat cancer. It essentially harnesses the body's immune system to fight against cancer. Previous literature suggests that cancer vaccines designed against a specific tumor antigen have been efficiently utilized to trigger immune responses against tumor cells. Despite the preliminary evidence in animal models, low immunogenicity is one of the major hurdles in the development of vaccines in humans. In order to surmount this obstacle, several approaches including the use of an "ideal" tumor antigen, appropriate delivery techniques, immune boosting strategies with co-stimulatory molecules are being explored.

The purpose of this study was to develop "bacteriomimetic nanoparticles" to enhance adaptive cell-mediated immune responses ($CD4^+$ and $CD8^+$ T cell responses) against tumor antigen as a therapeutic option of cancer treatment.

NPs were prepared by the solid/oil/water solvent evaporation method using an ultrasonic processor UP200H system (Hielscher Ultrasonics GmbH, Germany). We used membrane preparations of the 4T1 mouse mammary cancer cell line as a tumor antigen and CpG ODN's as a "bactriomimetic" stimulant. Balb/c female mice (6-8 wk) were pre-immunized by intraperitoneal injection (IP) with CpG followed by secondary immunization using respective NPs encapsulated with the membrane antigen preparation 14 days before tumor challenge. Subsequently, mice were challenged with

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10^5 tumor cells intravenously (IV). Primary tumor size was measured using a vernier caliper. Mice were sacrificed and tumors were harvested at days 3, 7 and 14 respectively. Lungs were also harvested at similar time intervals to determine formation of lung lesions. Furthermore, CD4 + and CD8 + T cell responses were measured in lung and spleen using flow cytometry.

Tumor size data suggests regression in tumor size in animals immunized with CpG coated NPs containing tumor antigen (CpG-NP-Tag). No differences in lung lesions were found. Cytometry analysis demonstrated increased CD4 + (helper) and CD8 + (cytotoxic) T cell response emphasizing enhanced immunogenicity against cancer cells (Fig. 3).

Key Words: Breast cancer, Cancer vaccine, Cancer immunotherapy.

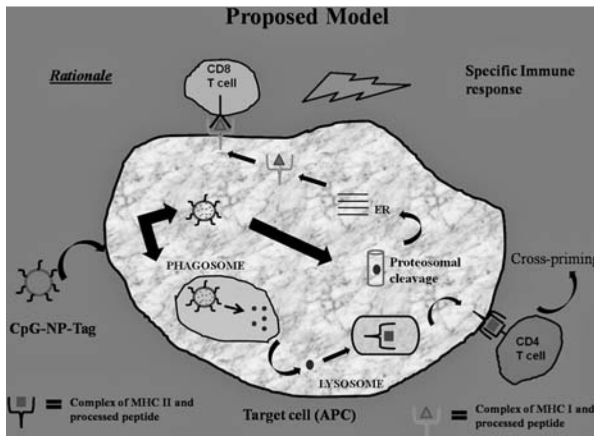

FIGURE 3. Proposed model.

Assessment of Cellular Immune Responses Following Poxvirus Based Cancer Immunotherapies

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BN ImmunoTherapeutics Inc. is developing cancer immunotherapies using poxvirus based vectors that encode heterologous cancer antigens. The MVA-BN®-vector is a highly attenuated vaccinia virus that is non-replicating in humans and has been shown to be an immunogenic vaccine. MVA-BN®-based immunotherapy vectors have been tested in proof-of-concept animal models as well as in early stage clinical settings. The MVA-BN®-vector has been utilized to express tumor specific proteins for breast cancer (MVA-BN®-HER2) and prostate cancer (MVA-BN®-PRO).

MVA-BN®-HER2 utilizes a poxviral vector that encodes a modified and truncated form of the HER-2 epidermal growth factor receptor (HER-2 extracellular domain plus 2 tetanus toxoid peptide epitopes), and has been tested in metastatic and adjuvant breast cancer settings. MVA-BN®-PRO has been engineered to encode prostate specific antigen (PSA) and prostate acid phosphatase (PAP) proteins and was evaluated in an open-label multicenter dosing evaluation clinical trial in non-metastatic castration resistant prostate cancer (CRPC).

Immune evaluation of patients treated with MVA-BN®-HER2 and MVA-BN®-PRO enrolled in phase I clinical trials was performed to determine the relevant immune parameters that correlate with clinical benefit. A variety of cellular immune response assays were performed. These included the IFN-γ ELISPOT assay, the CFSE-based proliferation assay, as well as a flow cytometry based intracellular cytokine secretion and degranulation assay. Immune monitoring revealed the induction of cellular immune responses specific to the transgene encoded by the vector in the majority of patients treated with MVA-BN®-HER2 and MVA-BN®-PRO. The presence of a pre-existing immune response to MVA did not impair the induction of transgene specific immune responses. Additionally, vaccine-induced determinant spreading was evident in tumor-bearing patients treated with MVA-BN®-HER2 and MVA-BN®-PRO. The therapies were well tolerated with no dose-limiting toxicities or serious adverse events reported. The data suggest that MVA-BN®-HER2 and MVA-BN®-PRO are well-tolerated, immunogenic, and support going forward with larger efficacy trials.

Key Words: Cancer vaccine, Cellular immunity, Cytokine.

Therapeutic Effect of Cancer Stem Cell-based Vaccine

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The isolation of human cancer stem cells (CSC) represents a new paradigm for the development of cancer treatments. So far, the majority of the stem cell studies have been confined to human tumors inoculated into severely immunosuppressed hosts (e.g. SCID mice), where adaptive immune responses are absent, and such hosts are therefore not suitable for the immunologic evaluation of CSCs. The lack of immunocompetent syngeneic animal tumor models where stem cells can be isolated has been the primary obstacle to evaluating the immunogenicity of CSCs. We recently reported the identification of CSC-enriched populations using ALDEFLUOR/ALDH as a marker in murine melanoma D5 and squamous cell cancer SCC7, and evaluated their immunogenicity in two genetically distinct syngeneic immunocompetent hosts. Enriched CSCs are immunogenic and significantly more effective as an antigen source in inducing protective anti-tumor immunity than unsorted tumor cells or purified non-CSCs. We further found that selective targeting of CSCs by CSC-primed antibodies and T cells represents the mechanisms involved in CSC vaccine-conferred protective immunity. If a CSC vaccine is to be clinically relevant, it needs to be evaluated in a therapeutic model. In this study, we examined two models for this purpose. The first model involves the treatment of micrometastatic disease. We initiated vaccination 24 hours after s.c. inoculation of D5 tumor cells in the syngeneic immunocompetent host (B6 mice), and repeated the vaccination one week later. Comparison was made among the group with PBS-injected controls; mice vaccinated with dendritic cells (DC) pulsed with the lysate of ALDHhigh D5 cells (CSC-TPDC) vs. ALDHlow D5 cells (ALDHlow -TPDC). We found that CSC-TPDC inhibited the tumor growth more than ALDHlow-TPDC and PBS controls, and significantly prolonged the survival of the tumor-bearing mice. The second model involves the treatment of established tumors using CSC-TPDC vaccine as an additional strategy to radiation therapy (RT). Day 7 D5 sc tumors were treated with localized RT with repeat treatments on D8. Vaccine therapy commenced on D8. This combination therapy was repeated twice with one week apart. CSC-TPDC plus RT mediated D5 tumor regression more than ALDHlow-TPDC + RT, RT alone and PBS controls, and prolonged the survival of the tumor-bearing mice. Importantly, we observed metastasis of the D5 tumors to the lung in all the groups except for the one treated with CSC-TPDC plus RT. These results provide a rationale for the development of immunological approaches for the therapy of cancer by targeting cancer stem cells. The findings may help develop novel immunological approaches for cancer treatment by utilizing an autologous cancer stem cell-based vaccine.

Key Words: Cancer vaccine, Dendritic cell.

Novel Clinical Research Immune Monitoring Model of EX Vivo Induction of MRNA

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Immune monitoring is an important first step for the analysis of immune modifying drugs as well as immunotherapy. Although the

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monitoring of humoral immunity can be easily performed by quantifying the titer of specific IgG in serum, the analysis of cell-mediated immunity is not yet standardized, due mainly to the complexity of cellular immunity and correlates of clinical efficacy as well as technical variations of cell isolation/culture and stability/functionality of blood samples. To address the latter technical problems, we first developed stimulant-containing heparin-coated vacuum blood collection tubes. Examples of such stimulants are phytohemagglutinin-L (PHA), heat aggregated IgG (immune complex), lipopolysaccharide (LPS) (toll-like receptor agonist), recombinant human IL2 (rIL2), mouse monoclonal antibody against human α/β chain of T cell receptor, peptide vaccine antigen(s) itself and others, with or without combinations of various immune-modifying drugs. Since each tube takes approximately 1.5 mL of whole blood, only 12 mL of blood is enough for 7 stimulants and 1 control (total 8 tubes). Moreover, blood cells are immediately stimulated with these agents without any time lag, and without cell isolation procedures. We then developed a unique transportation box, where blood samples were maintained at 37 degreeC for 4 hours, then refrigerated overnight. Thus, incubation can be done during transportation. Moreover, because the box contains a temperature logger, post-blood draw temperature profile can be assessed in each case. The blood was then used to quantify various immune function-associated mRNA by the method we reported previously (J Immunol Methods. 363:95-100, 2010). Since whole blood contains multiple types of leukocytes, specific population of cells can be analyzed for functionality by carefully choosing right combinations of stimulant(s) and mRNAs. After phlebotomists were trained appropriately, the system was successfully implemented as an exploratory first step in a multicenter clinical trial of cancer peptide vaccine, and target mRNAs were quantified. Understanding of cellular immunology is evolving very rapidly, and various potential biomarkers are identified that can be used as correlates of clinical efficacy during immunotherapy. In order to validate clinical applications of such new knowledge, the system described in this study is expected to be a leading research model in the future.

Key Words: Engineering, Biomarker, Cellular immunity.

Discovery of Unique Biomarkers Which Predict Clinical Responses to Dendritic Cell Vaccine

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Dendritic cell (DC) vaccine therapy is a new weapon against cancer, however, it requires labor-intensive cell preparation procedures, leading to one of expensive therapy options. If patient likely respond to DC vaccine were predicted, this therapy might be considered as a standard care. We demonstrated our preliminary results last year, where 14 different leukocyte-function-associated (IFNG, TNFSF1, 2, and 5, IL2, 8, and 10, TGFB, CTLA4, PDCD1, FOXP3, GMCSF, VEGF, and CXCL3) and 2 control mRNAs (ACTB, B2M) were quantified after ex vivo stimulation with 8 different agents (phytohemagglutinin (PHA), heat aggregated IgG (HAG), zymosan A (ZA), recombinant human IL2 (rIL2) and interferon (rIFNα2b), monoclonal antibody against α/β chain of T cell receptor (TCR), picibanil, and phosphate buffered saline (PBS). Ex vivo stimulation was performed at 37 degreeC for only 4 hours using heparinized whole blood obtained from patients before DC vaccine therapy. Since each reaction used 0.06 mL of whole blood, the volume needed for this assay was as small as 1.5 mL, even in triplicate. This year, we added 21 more advanced cancer patients (total 47 patients) with a variety of cancer types, and the clinical outcome (PD, SD, and PR) was determined by the RECIST criteria, without knowing mRNA data. The number of mRNA preparation/cDNA synthesis was 1,128 (8 stimulants x 3 (triplicate) x 47 (patients). The fold increase (FI) was calculated using the values of PBS. FI of ACTB and B2M was not different among 3 groups, and all subjects showed at least 1 mRNA induction, suggesting that the assay condition was appropriate and functionality of blood samples was maintained. Significant difference between PD (n = 21) and PR (n = 11) were found in PHA-induced CTLA4, PDCD1, IL8, HAG-induced IL8, CXCL3, Picibanil-induced IFNG, anti-TCR-induced IL8, and rIFNα2b-induced IL10 ($P < 0.05$). When SD and PR were combined, only PHA- and HAG-induced IL8 were significant ($P = 0.03$, 0.04, respectively). More importantly, using multivariate discriminant analysis, various prediction formulas were developed. When 18 parameters were used, which were derived from 9 PHA-, 5 ZA-, 1 each of HAG- and rIL2-, and 2 TCR-induced mRNAs, the prediction of PD and SD + PR were 100% and 93% in both the first (n = 26) and the second set (n = 21) of patients, respectively. In order to achieve such high prediction rate, single gene or single stimulant was not enough, and the combinations of multiple immune components were required. This is reasonable, because clinical outcome is dependent on the balance among various immune functions in each patient at the time of DC vaccine therapy. This formula will be used to identify appropriate DC vaccine candidates and non-candidates in the future.

Key Words: Advanced cancer, DC-based vaccine, Biomarker.

A BI-institutional Pilot Study of Peptide-based Vaccines in Combination With Poly ICLC in Patients With Who Grade 2 Low-grade Glioma

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Adult patients with WHO grade 2 low-grade glioma (LGG) have a significant risk of tumor progression despite treatment with surgery or surgery followed by radiation therapy (RT) and/or chemotherapy, and most patients eventually die of the disease. Because patients with LGGs are likely not to be as immuno-compromised as patients with high-grade glioma (HGG), they may exhibit greater immunological response to and benefit from the vaccines. Further, the generally mild toxicity of vaccines may help maintain a higher quality of life than is experienced with current cancer therapy. Based on promising data from our phase I/II study targeting multiple glioma-associated antigen (GAA) epitopes in patients with recurrent HGGs, we initiated a pilot study of subcutaneous vaccinations with synthetic peptides for GAA epitopes emulsified in Montanide-ISA-51 every 3 weeks for 8 courses as well as intramuscular administration of poly-ICLC in HLA-A2 + patients with: newly diagnosed high-risk WHO grade 2 LGG without prior RT (Cohort 1); newly diagnosed high-risk LGG with prior RT (Cohort 2); or recurrent LGG (Cohort 3). Primary endpoints were safety and CD8 + T-cell responses against vaccine-targeted GAAs, assessed by ELISPOT assays. Treatment response was evaluated clinically and by MRI. GAAs for these peptides are IL-13Rα2, EphA2, WT1, and Survivin. A pan-HLA-DR tetanus toxoid peptide (TetA830) was included to enhance general helper CD4 + T-cell response. To date, 12, 1, and 10 patients have been enrolled in Cohorts 1, 2, and 3, respectively. No regimen-limiting toxicity has been encountered except for one case with Grade 3 fever (Cohort 1).

ELISPOT assays, completed in 7 and 1 patients in Cohorts 1 and 2, respectively, demonstrated robust and sustained IFN-γ (type-1) responses against at least 3 of the GAA epitopes in all cases, while IL-5 (type-2) responses were absent or transient in all cases. The magnitude of the IFN-γ ELISPOT responses in this study is significantly higher than that observed in our previous phase I/II study in HGG patients. One case demonstrated evidence of epitope-spreading based on the post-vaccine induction of IFN-γ ELISPOT responses against GAAs that were not included in the vaccines. Currently, 5 of 10, 1 of 1, and 2 of 8 patients in Cohorts 1, 2, and 3, respectively, who received all 8 vaccinations are stable (median follow-up of 16.2 mo). Our preliminary results demonstrate the regimen in these patients is well tolerated and induces a robust type-1 anti-GAA T-cell response.

Key Words: Glioblastoma, Cancer vaccine, Th1/Th2 polarization.

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Evaluation of HER-2 Specific Humoral Immune Responses in Breast Cancer Patients Treated With MVA-BN®-HER2

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MVA-BN®-HER2 is a poxviral vector that encodes the extracellular domain of human HER-2 as well as two universal tetanus toxin T cell epitopes. Preclinical data have demonstrated MVA-BN®-HER2 to be immunogenic, inducing strong anti-tumor activity (Mandl et al, iSBTC 2010). MVA-BN®-HER2 has also been evaluated in various phase I safety and immunogenicity trials, with 30 HER-2-positive breast cancer patients being tested in the metastatic setting and 15 patients following adjuvant therapy. Previous immunological monitoring of MVA-BN®-HER2 treated patient samples revealed that treatment was able to break tolerance against HER-2 in the adjuvant and metastatic settings, inducing humoral and/or T-cell responses in the majority of the patients (Legrand et al, iSBTC 2010 and Owen et al, SITC 2011).

Extended analysis of humoral responses was performed in patients receiving MVA-BN®-HER2 to determine the relevant immune parameters that correlate with clinical benefit. The generation of HER-2 transgene and MVA vector specific antibody responses was assessed with the ELISA IgG titer assay. The breadth of the anti-tumor response was determined using a peptide array comprised of 7590 peptides derived from 46 breast cancer tumor associated antigens (TAA) including HER-2. In addition, the role of vaccine induced HER-2 specific antibodies in eliciting functional anti-tumor activity is being evaluated.

Overall, it was observed that qualitatively different anti-HER-2 antibody responses were induced in patients treated with MVA-BN®-HER2. The peptide array assay revealed that repeated treatment was accompanied by a broadening of the anti-HER-2 humoral response as well as epitope spreading to other TAAs. Strong responses to 15 TAA proteins were detected in at least 12 out of the 30 tested patients. In addition, 42 out of the 7590 total evaluated peptides were identified as being immunodominant. Importantly, the presence of a pre-existing immune response to the MVA vector did not impair the induction of transgene specific immune responses. The broadening of immune responses to non-HER-2 TAAs suggests that the MVA-BN®-HER2-mediated immune activation results in anti-tumor activity. Taken together, these data support MVA-BN®-HER2 treatment to be a potent activator of humoral immune responses in both the metastatic and adjuvant settings.

Key Words: Cancer vaccine, Antibody response, Tumor associated antigen.

Tumor-Derived Alpha-fetoprotein Impairs the Differentiation of Human Dendritic Cells

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Within the past thirty years, the incidence and mortality rates for hepatocellular carcinoma (HCC) have tripled in the United States. Numerous immune suppressive mechanisms are thought to drive HCC development and therapeutic resistance. In order to improve clinical outcomes for HCC patients, identifying and counteracting these factors will therefore be crucial. It has been proposed that alpha-fetoprotein (AFP), an oncofetal antigen that is the most abundant serum protein in the fetus and is re-expressed by HCC tumors, plays an immunoregulatory role. In this study, we investigated the effect of AFP of dendritic cell (DC) differentiation, maturation, and function. Human peripheral blood monocytes were cultured with GM-CSF and IL-4 for five days in the presence or absence of cord blood-derived normal AFP (nAFP) or HCC tumor-derived AFP (tAFP). Although the nAFP and tAFP isoforms only differ at one carbohydrate group, we unexpectedly observed that tAFP, but not nAFP, interferes with the differentiation of monocytes into dendritic cells (DC) in a dose-dependent manner. Despite high viability, these tAFP-conditioned DC expressed diminished levels of several DC maturation markers and retained a monocyte-like morphology. Moreover, this effect could not be abrogated by simultaneous culture with nAFP, heat inactivation of tAFP, or subsequent maturation of cells with IFN-gamma and LPS. Ongoing studies are addressing the allostimulatory function and cytokine profile of tAFP-conditioned DC, as well as the ability of tAFP + HCC patient serum or cell culture supernatants to inhibit DC differentiation. These data suggest that elevated levels of serum tAFP in HCC patients may inhibit endogenous DC differentiation, thereby supporting clinical observations that the peripheral blood of these patients contains reduced frequencies of myeloid DC. Novel therapeutic approaches that antagonize or circumvent tAFP and other regulatory circuits will be critical to optimizing clinical outcomes for HCC patients.

Key Words: DC-based vaccine, Dendritic cell, Tumor associated antigen.

Peptide Vaccine Therapy for Childhood Gliomas: Interim Results of a Pilot Study

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Diffuse brainstem gliomas, other malignant astrocytomas and multiply recurrent low-grade gliomas carry a poor prognosis with current treatments. We initiated a pilot trial of subcutaneous vaccinations with peptides for glioma association antigen (GAA) epitopes emulsified in Montanide-ISA-51 given every 3 weeks for 8 courses along with intramuscular injections of poly-ICLC in HLA-A2 + children with newly diagnosed brainstem gliomas, high-grade gliomas, or recurrent gliomas. GAAs were EphA2, IL13Rα2, and survivin, three proteins that we demonstrated were overexpressed in childhood gliomas. Primary endpoints were safety and T cell responses against vaccine-targeted GAAs. 28 children have been enrolled and assessed for response to date, 16 with newly diagnosed BSG, 5 with newly diagnosed HGG, 4 with recurrent low-grade gliomas and 3 with recurrent HGGs. No dose-limiting non-CNS toxicity has been encountered. Seven children had immunological pseudoprogression, which was symptomatic in 6, but responsive to corticosteroids. Among 24 patients evaluable for response, 3 had rapidly progressive disease, 16 had stable disease for > 2 cycles, 3 had PRs, 1 had an MR, and 1 had prolonged disease-free status after surgery. ELISPOT analysis, completed in 13 children, showed GAA responses in 11, most commonly to IL13Rα2. Peptide vaccination in children with gliomas is generally well tolerated, although distinguishing pseudoprogression from true progression can be challenging. Immunological and clinical evidence of activity has been obtained. More extensive analyses of efficacy in a multi-institutional context are warranted.

Key Words: Glioblastoma, Cancer vaccine, Cancer immunotherapy.

Identification of a Novel Age-related Dendritic Cell Deficiency and its Impact on Tumor Immunity

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Immune responses progressively wane during aging (i.e. immune senescence), posing significant challenges to protect and treat a growing elderly population from diseases, including cancer. Our previous work demonstrated that older animals exhibited impaired anti-tumor immune responses and diminished CD4 T cell effector

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differentiation in the context of OX40 costimulation. Furthermore, these impaired immune responses were attributed to age-related deficiencies in the host environment. Signals from dendritic cells (DCs) strongly influence immune responses; therefore, we hypothesized that the observed age-impaired immune responses were the result of deficient DCs in the priming microenvironment. We assessed a number of various DC subsets found in the LN following soluble antigen challenge in the context of OX40 costimulation. There was no significant age-related difference in the number and function of CD11c + DCs within 18 hours of immunization. We, however, observed a significant decrease in the number of a migratory inflammatory DC population (CD11c + CD11b + Ly6C +) found in the draining LNs of older animals starting 24 hours after immunization. Additional experiments determined that this inflammatory DC subset was critical for the effector differentiation of antigen-specific CD4 T cells. Finally, in a tumor model, older tumor-bearing mice experienced a decrease in the numbers of this inflammatory DC population in the tumor-draining LNs shortly after tumor challenge that correlated with a decrease in anti-tumor immunity. Thus, we have identified a novel age-related deficiency in the recruitment or retention of an inflammatory DC population, shown to produce IL-12 and induce Th1 responses. These finding could have implications in the effectiveness of immunotherapies, such as tumor vaccines, in elderly cancer patients.

Key Words: Tumor immunity, Lymph node, Dendritic cell.

Memory CD8 + T Cells, Secondary Expansion and Self-help: How Heterologous Prime-boost Vaccination Circumvents the Need for CD4 + T Cell Help and CD40-CD40L-signaling

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Heterologous prime-boost vaccination regimens are appreciated to elicit a more potent antigen-specific CD8 + T cell response than multiple immunizations using the same vaccine vector. Yet aside from avoidance of vector-neutralizing antibodies, the mechanisms responsible for the superiority of heterologous prime-boost immunization remain unclear. We investigated the requirement for CD4 + T cell help, CD40-CD40L signaling and systemic inflammation for the secondary expansion of memory CD8 + T cells. Following either homologous or heterologous prime-boost vaccination, memory CD8 + T cell secondary expansion was independent of CD4 + T cell help. Alternatively, CD40L signaling was required to maximize CD8 + secondary expansion following homologous, but not heterologous, prime-boost vaccination. Dependence on CD40 signaling correlated with accelerated vaccine clearance and decreased inflammation following homologous secondary immunization. Antibiotic treatment during heterologous boost recapitulated the CD40-CD40L dependence observed after homologous boost. Together, our studies reveal a distinct population of CD40L-expressing memory CD8 + T cells that are essential for maximizing expansion of the antigen-specific memory CD8 + T cell pool when the innate inflammatory response is limited. Conversely, boosting with a heterologous vaccine vector prolongs the duration and magnitude of the inflammatory response and promotes memory CD8 + T cell secondary expansion independent of CD4 + T cell help or CD40-CD40L signaling.

Key Words: Memory CD8 + T cells, Immunization, Active immunotherapy.

Phase IB Study on Intravenous Synthetic MRNA Electroporated Dendritic Cell Immunotherapy in Pretreated Advanced Melanoma Patients

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Purpose: Autologous monocyte-derived dendritic cells (DCs) electroporated with synthetic messenger RNA (smRNA) encoding CD40 ligand, a constitutively active Toll-like receptor 4 and CD70, together with smRNA encoding fusion-proteins of a HLA-class II targeting signal (DC-LAMP) and a melanoma-associated antigen (either MAGE-A3, MAGE-C2, tyrosinase or gp100) (TriMixDC-MEL) are immunogenic and can be safely administered by the intradermal (id) route. This clinical trial investigates the combined id and intravenous (iv) administration of TriMixDC-MEL in patients with pretreated advanced melanoma.
Experimental Design: Twenty-four million viable DCs are administered by 4 biweekly id/iv infusions, and a 5th administration on week 16. The number of iv-administered DCs is escalated from 4×10^6, over 12×10^6, and 20×10^6 to 24×10^6 in sequentially treated patient cohorts. Tumor response assessments (by RECIST) are performed by [(18)F]fluorodeoxyglucose-positron emission tomography/computed tomography at baseline and in weeks 8, 16 and 24. Immune responses are assessed by analysis of antigen-specific skin infiltrating lymphocytes (SKILs) at an id-injection site and in the blood.
Results: Fifteen patients with advanced and pretreated melanoma tolerated administration of TriMixDC-MEL well. Two patients achieved a complete response and two patients a partial response. All objective tumor responders and one patient with a disease stabilization are progression-free after a follow-up of respectively 13 + , 17 + , 18 + , 22 + and 23 + months. Post-therapy antigen-specific SKILs were documented in 6/12 patients, and antigen-specific CD8 + T-cells were detected in the blood of 4/5 patients.
Conclusions: Cellular immunotherapy with TriMixDC-MEL is safe and immunogenic. Anti-tumor activity with durable disease control is observed across the investigated iv-dose levels.

Key Words: Melanoma immunotherapy, Cancer vaccine, DC-based vaccine.

Intratumoral and Intranodal Administration of Trimix and Antigen MRNA Results in Effective Anti-tumor Immunity

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The use of tumor-associated antigen (TAA) mRNA for therapeutic purposes is under active investigation. To be effective, mRNA vaccines need to deliver activation stimuli in addition to TAAs to dendritic cells (DC). In this study, we evaluated whether intranodal and intratumoral delivery of TAA mRNA together with TriMix, a mix of mRNA encoding CD40 ligand, constitutive active Toll-like receptor 4 and CD70, results in the in situ modification and maturation of DCs, hence, priming of TAA-specific T cells.
We showed selective uptake and translation of mRNA in vivo by lymph node and tumor resident CD11c cells. This process was hampered by codelivery of classical maturation stimuli but not by TriMix mRNA. Importantly, TriMix mRNA induced a T-cell-attracting and stimulatory environment, including recruitment of antigen-specific CD4- and CD8-T cells and CTLs against various TAAs. In several mouse tumor models, mRNA vaccination was as efficient in CTL induction and therapy response as vaccination with mRNA-electroporated DCs. Intratumoral administration of TriMix mRNA only induced the migration of tumor-resident DC towards the draining lymph nodes and induced TAA-specific T cells.
Together, our findings suggest that intranodal and intratumoral administration of mRNA encoding immunomodulating molecules together with TAA mRNA is a promising vaccination strategy.

Key Words: Tumor immunity, Cancer vaccine.

Therapeutic Systemic Vaccination in Subjects With Pre-invasive HPV Disease is Associated With Changes in the Immune Cells Infiltrating the Target Tissue

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In women with preinvasive cervical lesions (high grade dysplasia, CIN2/3), nearly all caused by human papillomavirus (HPV), systemic T cell responses to HPV antigens are modest, requiring ex vivo manipulation to be detected. However, in the cervical mucosa, immune cell infiltrates do predict regression. In persistent lesions, T cell infiltrates are restricted to lesional stroma, while lesion regression is associated with intraepithelial CD8 infiltrates. Intraepithelial HPV lesions are associated with a shift in the composition of tissue immune cell subsets. Whereas normal cervix T cells exhibit a effector memory (TEM) phenotype, and have a CD8:Treg ratio of approximately 6:1, T cells in persistent dysplasia are comprised of a greater number and percentage of cells with a central memory (TCM) phenotype, and accumulation of many more Treg cells (CD8:Treg 3:1). We have been enrolling healthy subjects with HPV16 + CIN2/3 on a prospective trial testing priming vaccinations with a DNA construct targeting HPV16 E7 (wk 0 and 4), and boost vaccination with a recombinant vaccinia construct targeting HPV16 and 18 E6 and E7 (TA-HPV) (wk8), to enhance the host response to the lesions, which persistently express the E6 and E7 antigens. Individuals are being analyzed pre- and post-vaccination, not only for systemic responses, but also for changes in the lesions, by comparing diagnostic biopsies obtained pre-intervention to post-vaccination resection specimens at week 15. Within-subject comparisons of pre- and post-vaccination tissue show a shift back towards TEM cells, and towards normalization of the CD8:Treg ratio. In contrast to an observational cohort of unvaccinated subjects with CIN2/3 followed over the same study window, tissue lymphocytes in vaccinated subjects are Ki67 + , consistent with proliferation via activation via cognate antigen. Within-subject comparisons show increases in CD8 + infiltrates in lesional mucosa significantly greater in vaccinated than in unvaccinated subjects. Stromal lymphocytes in vaccinated subjects are often organized in either lymphoid aggregates, or in frank germinal centers. These findings indicate that systemic vaccination with a heterologous DNA prime, recombinant vaccinia boost regimen is followed by accumulation of proliferating TEM in the target lesion. Studies to quantitate T cell receptor diversity are ongoing. We have been pursuing strategies to enhance access to lesional epithelium, using local application of TLR agonists. Our findings support future trial designs that incorporate strategies to enhance the access of effector T cells into lesional epithelium.

Key Words: Cancer immunotherapy, HPV, Tumor infiltration lymphocytes.

Tumor Autophagosome-based Cancer Vaccine Combined Immunotherapy With Anti-OX40 Provides Therapeutic Immunity Against Established Breast Cancer

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Our group has shown that tumor macroautophagy is critical for antigen delivery to professional APCs and for the generation of an effective anti-tumor immune response. Exploiting these observations, we showed that vaccination with tumor-derived autophagosomes (DRibbles) provides cross-protection against a panel of syngeneic MCA sarcomas while irradiated whole tumor vaccine was ineffective, breaking a 50-year paradigm. Data supports that this vaccine contains a broad repertoire of antigen as well as the abundance of both damage-associated molecular pattern molecules and ligands for CLEC9A to promote crosspresentation. In a recent set of three independent experiments, an intranodal 4T1 DRibble vaccine provided a significant increase in the survival of mice bearing 5-day orthotopic 4T1 tumors ($P < 0.05$ n = 15 mice/group) that did not occur with a whole cell 4T1 vaccine. Based on previous studies and the difficulty of treating mice in this therapeutic model, anti-OX40 was combined to augment the vaccine-induced T cell response. While anti-OX40 alone had the same impact on the survival of mice bearing 5-day tumors as the 4T1 DRibble vaccine, combination of the 4T1 DRibble vaccine and anti-OX40 significantly enhanced the survival of mice compared to vaccine alone ($P < 0.05$ n = 15 mice/group) which correlated with a > 50% increase in the ratio of proliferating CD8 + T cells to CD4 + Treg cells (2 independent experiments n = 6 mice/group).
In all of the preceding studies, the vaccines were syngeneic to the host. Since the clinical translation of these findings would be accelerated if the therapeutic effect could be replicated in an allogeneic setting, we utilized three breast tumor cell lines on three different H2 backgrounds (H2b, H2d and H2q) to test the therapeutic potential of a DRibble vaccine in a 5-9 day established tumor model using a criss-cross experimental design. Whole cell vaccination with irradiated “allogeneic” or “syngeneic” tumors failed to provide significant therapeutic efficacy against 5-, 9- or 7-day established 4T1, FAT or C57mg tumors in BALB/C, FVB/N or C57BL/6 mice respectively (data from 9 independent experiments). In striking contrast, vaccination with either the syngeneic DRibble vaccine or one of the two allogeneic DRibble vaccines provided therapeutic effects in all combinations studied ($P < 0.05$ n = 12-24/group). These results demonstrated a shift in our understanding of how to prime an effective anti-tumor immune response and provide insights into novel immunotherapy strategies that might be employed to more effectively treat patients with cancer

Supported by ACS #PF-12-243-01 and Providence Medical Foundation.
Key Words: Therapeutic vaccine, Breast cancer, Cancer immunotherapy.

Induction of MAGE-A6-Specific CD8 + T-cell Responses Using MAGE-A6 and Mycoplasma Penetrans HF-2 Permease-derived Peptides

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A promising vaccine strategy for treatment of cancer involves the use of synthetic peptides derived from a tumor-associated antigen, and which encompass multiple epitopes capable of stimulating both CD4 + and CD8 + anti-tumor T-cell responses. Previously, we have reported that a MAGE-A6-derived peptide (MAGE-A6.172-187) and its highly immunogenic and cross-reactive homologue derived from Mycoplasma penetrans HF-2 permease (HF-2.216-229) are promiscuously presented by many HLA-DR alleles, and are capable of stimulating MAGE-A6-specific CD4 + T-cell responses in the majority of healthy donors and melanoma patients tested. Here, we investigated whether the same peptides could also stimulate MAGE-A6-specific CD8 + T cell responses. Using cells isolated from HLA-A*0201 (HLA-A2) + healthy individuals and melanoma patients, we showed that MAGE-A6.172-187 and, particularly, HF-2.216-229 induced cross-reactive memory CD8 + T-cell responses capable of recognizing HLA-matched, MAGE-A6 + tumor targets. Their shared immunogenicity was partially attributed to internal MAGE-A6.176-185 and HF-2.220-229 epitopes within MAGE-A6.172-187 and HF-2.216-229, respectively, which were targets for MAGE-A6.172-187 and HF-2.216-229-stimulated CD8 + T-cell responders. The two peptides induced a number of different CD8 + T cell clones, as shown by T-cell receptor Vβ segment staining. Some of the clones were uniquely reactive to one peptide and some were reactive to both peptides. These data indicate that the MAGE-A6.172-187 and HF-2.216-229 homology is extended beyond HLA-DR promiscuity and CD4 + T cell stimulation, and that they are also capable of inducing MAGE-A6-specific polyclonal CD8 + T cell responses. This newly-described property of these peptides further confirms their potential in patient vaccination and/or monitoring of cancer patients.

Key Words: Cancer immunotherapy, CD8 + T cells, Tumor associated antigen.

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Alarmin HMGN1 Promotes Antitumor Immunity

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Alarmins are endogenous mediators that rapidly become available in peripheral tissues in response to danger signals and are capable of enhancing the induction of innate and adaptive immune responses by promoting the recruitment and maturation of antigen presenting cells (APCs). We have previously shown that high-mobility group nucleosome-binding protein 1 (HMGN1) is an alarmin that contributes to the development of antigen-specific immune responses. Interestingly, administration of exogenous HMGN1 with an antigen selectively promotes the development of antigen-specific Th1 immune response. In this study, we investigated whether HMGN1 played an important role in the generation of antitumor immunity. Inoculation of EG7, a mouse thymoma transfected to overexpress OVA, into Hmgn1-/- and littermate-matched Hmgn1 + / + mice revealed that the tumor grew much faster in Hmgn1-/- mice than in Hmgn1 + / + mice. In addition, EG7-bearing Hmgn1-/- mice had fewer splenic OVA-specific CD8 cells, suggesting that endogenous HMGN1 contributed to the development of antitumor immune responses. To determine whether exogenous HMGN1 could also enhance anti-tumor defense, we inoculated EG7-N1, an HMGN1-expressing EG7 tumor cell line, and parental EG7 into C57BL/6 mice. Monitoring the growth of implanted tumors showed that EG7-N1 tumor grew slower than EG7 tumors in the mice, while both cell lines proliferated equally in vitro, suggesting that the murine immune system could be "awakened" by the HMGN1 expressed by the tumors. These results indicate that HMGN1 contributes to the development of antitumor immunity. To verify the capability of the alarmin HMGN1 to enhance antitumor immune responses, we constructed a series of eukaryotic expressing plasmids encoding the genes of HMGN1, gp100 (a murine melanoma-associated antigens), or HMGN1-gp100 fusion protein, and used these plasmids as DNA vaccines to determine whether vaccination with HMGN1-gp100 fusion gene could promote the generation of anti-melanoma immunity. C57BL/6 mice vaccinated by genegun with various plasmids were subcutaneously implanted with B16F1 melanoma (2×10^4/mouse) and monitored for tumor growth for 4 weeks. A significant tumor growth inhibition was observed in mice vaccinated with HMGN1-gp100 plasmid, whereas control mice developed tumors. Further analysis revealed that T cells from mice immunized with HMGN1-gp100 plasmid generated a stronger gp100-specific cytotoxic activity. Overall, the data illustrate that HMGN1 contributes to the generation of antitumor immunity and suggest that the alarmin HMGN1 may be used as an effective tumor vaccine adjuvant.

Key Words: Cancer vaccine, Immunomodulation, Adjuvant.

Autophagy in Tumor Cells is Critical for Innate Immune Sensing of a Growing Tumor and Bridging to an Adaptive Immune Response

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Adaptive T cell responses are required for effective anti-tumor activity, and spontaneous T cell responses against tumors occur frequently. However, the mechanisms by which innate immune responses become induced in response to cancer, and how they can bridge to T cell priming against tumor antigens, are poorly defined. Our laboratory has recently shown that CD11c + cells produce IFN-β after tumor implantation and this IFN-β plays a critical role in the cross-priming of host CD8 + T cells in vivo. However, the sensing mechanism that mediates production of IFN-β by host DCs in response to tumor-derived products has remained unclear. Using specific gene targeted mice and in vitro experiments, we observed that tumor-derived DNA could trigger production of IFN-β by APCs, and mice deficient in the molecule STING (stimulator of IFN gene) were severely deficient in IFN-β production and T cell priming against tumors. Based on these observations, it has become critical to determine the mechanism by which tumor DNA might become delivered into the cytosol of DCs for cytosolic sensing of DNA and induction of IFN-β production. We hypothesized that the autophagosome might support delivery of tumor DNA into DCs by protecting it against degradation by DNase I and DNase II. To address this question, we inhibited autophagy of tumor cells either using a chemical approach (3-MA) or a genetic approach (shRNA knockdown of Beclin1). In fact, both 3-MA treatment and Beclin1 knockdown in B16.SIY melanoma cells led to significantly reduced anti-tumor T cell priming in vivo. These data suggest that autophagy induction in tumor cells might be linked to innate immune sensing of tumors. To evaluate whether tumor DNA transfer to host APCs could be detected in vivo, we stained tumor cells with the DNA-specific fluorescent dye DRAQ5 and implanted them into mice. Subsequently, tumor-infiltrating CD45 + CD11c + were analyzed by flow cytometry and using the Amnis ImageStream instrument. In fact, most these cells were positive for uptake of tumor DNA in a diffuse pattern, supporting the notion that tumor-derived DNA can end up in the appropriate cellular compartment for innate immune priming in vivo. These data suggest a possible mechanism by which tumor DNA might be acquired by host APCs in vivo and thereby lead to an adaptive T cell response against tumor-derived antigens in vivo.

Key Words: Immunogenic cell death, Dendritic cell, Innate immunity.

Phase I Trial of a Multi-epitope Pulsed Dendritic Cell Vaccine Targeting Cancer Stem Cells in Patients With Newly Diagnosed Glioblastoma

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This study evaluated the safety and immune responses to an autologous dendritic cell vaccine pulsed with class I peptides from tumor associated antigens (TAA) expressed on gliomas and over-expressed in their cancer stem cell population (ICT-107). TAA epitopes included HER2, TRP-2, gp100, MAGE-1, IL13Rα2, and AIM-2. HLA-A1 and/or HLA-A2 positive patients with glioblastoma (GBM) were eligible. Mononuclear cells from leukapheresis were differentiated into dendritic cells, pulsed with TAA peptides, and administered intradermally three times at two-week intervals. Twenty-one patients were enrolled with 17 newly diagnosed (ND-GBM) and three recurrent GBM patients and one brainstem glioma. TAA expression by qRT-PCR from fresh frozen tumor samples showed all patient tumors expressed at least three TAA with 75% expressing all six. CD8 + peptide specific CTL lines induced in vitro from normal donors to the HLA-A2 peptides, TRP2, gp100, HER2, and IL-13Ra2 showed killing of a CD133 + HLA-A2 + GBM neurosphere line (BTSC5). Correlations of increased PFS and OS with quantitative expression of MAGE1, AIM-2 were observed and a trend for longer survival was observed with gp100 and HER2 antigens. Target antigens gp100, HER1 and IL13Rα2 were down regulated in recurrent tumors from 4 HLA-A2 + patients. A decrease or absence of CD133 expression was seen in five patients who underwent a second resection. Immune response data on 15 newly diagnosed patients showed 33% responders. At a median follow up of 40.1 months, six of 16 ND-GBM patients showed no evidence of tumor recurrence. Median PFS in newly diagnosed patients was 16.9 months and median OS was 38.4 months. Expression of four ICT-107 targeted antigens in the pre-vaccine tumors correlated with prolonged overall survival and PFS in ND-GBM patients. The goal of targeting tumor antigens highly expressed on glioblastoma cancer stem cells is

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supported by the observation of decreased or absent CD133 expression in the recurrent areas of gadolinium-enhanced tumors.
Key Words: Glioblastoma, Cancer vaccine, DC-based vaccine.

IMMUNITY OF ONCOLYTIC VIRUSES

Effect of HCV Viraemia on NK Cells

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Besides the central role of NK cells in the pathogenesis of many viral infections, there is surprisingly little known about NK cells in HCV persistent viraemia. Imbalance of activating and inhibitory NK cell receptors contributes to the outcome of chronic HCV infection, although it has not been so well described. In the current study, we evaluated molecular profile of peripheral NK cells from healthy donors, chronically viraemic HCV-treatment-naïve patients and patients who spontaneously achieved virus eradication by whole-genome gene expression analysis (Affymetrix Human Gene ST.1.0 Arrays). FACS analysis of target activating cytoxicity receptors (NCR1, NCR2 and NCR3) was also assessed. Class comparison showed an up regulation of genes involved in the activation of effector functions of NK cells, such as GNLY, NKG2E, NKG2F and NKG2C in patients with spontaneous eradication of virus infection. On the contrary, an enhanced expression of genes involved in IFN signaling and IL-15 production, such as CXCL11, HLA-DOB, PTK, IFNβ and IFNα,was found to occur in chronically viraemic HCV patients. As previously described, FACS analysis shows an overexpression of NCRs in patients bearing chronic viraemia. Counter intuitively, neither a statically significant difference nor a trend between the group in comparisons were detected when NCRs were evaluated at the transcriptional level. Taken all together, our findings indicate that NK cells from chronic infected patients display an (reactive?) overexpression of the IFN pathways already at the transcriptional level with an activation of NCRs only gained at the protein level suggesting the interference of post-transcriptional mechanisms (eg. microRNA). Interestingly, other markers of immunoactivation, (i.e., immuno-effector function genes; GNLY) and other activating receptors are upregulated at the transcriptional level by the NK cells of patients who successfully eradicated viral infections. The evaluation of the corrisponding proteins expression on cell surface is currently ongoing. In conclusion we showed NK molecular signatures associated with HCV viraemia persistence. The functional interpretation of these data will need the integrated analysis of gene and protein expression as well as a detailed assessment of post-translational mechanisms.
Key Words: HAV/HBV/HCV infections, Immune escape, Innate immunity.

Combination Therapy of Intratumoral CRT/E7 Vaccinia Virus and Cisplatin Treatment Enhance the Antigen-specific T cell Immune Responses and Therapeutic Antitumor Effects

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Cervical cancer is the third most common female cancer in the world. Despite current treatment regimens, including radiation therapy and chemotherapy, stages 3 and 4 cervical cancers have a low five-year survival rate. Cancer immunotherapy has emerged as an alternative innovative therapy that may improve survival rates. Here we utilize a cervical cancer model and employ the chemotherapeutic agent cisplatin to generate an accumulation of CD11c + dendritic cells (DCs) in tumor loci along with a vaccinia virus vector expressing the chimeric protein calreticulin linked to HPV-16 E7 (CRT/E7-VV) to generate an E7-specific CD8 + T cell response. In this study, we explored the treatment of E7-expressing tumor-bearing mice with cisplatin in combination with intratumoral or intraperitoneal injection of CRT/E7-VV. We found that the combination of cisplatin and intratumoral injection of CRT/E7-VV significantly inhibited tumor growth and increased E7-specific CD8 + T cells in blood compared to cisplatin treated mice receiving intraperitoneal injection of CRT/E7-VV or mice treated with cisplatin alone. Furthermore, combination treatment with cisplatin and intratumoral CRT/E7-VV generated a systemic antitumoral and therapeutic response and reduces immunosuppressive myeloid-derived suppressor cells. The general methodology employed in this study may potentially be utilized as a platform to improve cancer immunotherapy. It will be important to continue examining possibilities for utilizing the treatment discussed here in combination with current treatment regimens for to improve survival of advanced stage cervical cancer.
Key Words: Cancer vaccine, Calreticulin, Chemotherapy.

Correlation Between Human and Oncolytic Vaccinia Virus Transcriptional Profile

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Introduction: Oncolytic viruses such as vaccinia virus (VACV) have emerged as an attractive strategy for cancer treatment. VACV replication efficiency is associated with increased cytotoxicity in vitro and with an improved therapeutic effect in mice. However, little is known about the influence of host factors on viral replication efficiency and permissiveness of a host cell line to infection and oncolysis. In this work, gene expression and replication efficiency of the recombinant VACV GLV-1h68 and wild type VACV isolates was determined in two autologous human melanoma cell lines. One major aim was to identify host genes that may modulate viral replication and thus permissiveness of cancer cell lines to oncolytic VACV therapy.
Methods: Host gene expression and viral gene expression in infected and uninfected cells were evaluated via a 36 k whole genome human array platform as well as a custom-made VACV array. Viral replication was determined by plaque assay analysis.
Results: The results demonstrate a probable correlation between VACV replication, viral early gene expression and the respective host response and thus a possible involvement of human host factors in viral early replication. Further we identified a set of human candidate genes as possible predictors for viral replication in an independent dataset.
Conclusion: Taken together our data suggest a probable correlation between viral replication, early gene expression and the respective host response. The identification of host factors that may play a role in viral replication could provide important information regarding host cell permissiveness to oncolytic virotherapy and thus facilitate the development of novel recombinant virus strains with improved therapeutic features.
Key Words: Engineering, Therapeutic vaccine, Melanoma.

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Oncolyic Myxoma Virus Delivery of Immunotherapeutic Genes to Brain Tumors

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Myxoma virus, a rabbit poxvirus, can efficiently infect and kill several mouse and human cancer cell lines. Recombinant viruses were previously engineered to express tdTomatoRed fluorescent protein (vMyx-tdTr) and mouse interleukin-15 (vMyx-IL15-tdTr). IL15 is a pro-inflamatory cytokine with a great potential for stimulating T lymphocytes and NK cells against cancer. It has been shown that coexpression of IL15 with the α subunit of IL15 receptor (IL15Rα) greatly enhances IL15 stability and function in vivo. Our previous studies have shown that earlier generation recombinant myxoma viruses (vMyx-tdTr and vMyx-IL15-tdTr), productively infect cancer cells in vitro, but have limited effect on tumors in vivo. In order to improve oncolytic efficacy of the virus in vivo, we engineered a new recombinant myxoma virus (vMyx-IL15Ra-tdTr), which expresses IL15Rα-IL15 fusion protein and tdTomatoRed fluorescent protein. Multi-step growth curves show productive infection of various cancer cell lines tested. Melanoma cell lines (B16-F10 and B16.SIY) are as permissive as the control cell line RK-13. Glioma cell lines (GL261 and GL261.SIY) are less permissive to myxoma infection. RK-13 cells infected with vMyx-IL15Ra-tdTr (MOI = 5) express and secrete the IL15Rα-IL15 fusion protein, as shown by Western blot analysis. Preliminary in vivo experiments, in which B16.SIY intracranial tumors were treated with 5×10^6 ffu vMyx-IL15Ra-tdTr i.t., showed a statistically significant survival benefit for the treated group compared to the PBS control (median survival of 18 vs. 12.5 d, respectively). We are currently continuing the in vitro evaluation of the novel recombinant vMyx-IL15Ra-tdTr and further testing it as a treatment for murine brain tumors in vivo. We hypothesize that the three virotherapeutic effects of the virus (oncolysis, delivery of IL15Rα-IL15, and immune activation from Toll like receptor-mediated inflammation) will augment the immunotherapeutic effects of T cell mediated tumor cell killing.

Key Words: Interleukin-15, Animal model, Immunotherapy.

IMMUNOTHERAPY COMBINATIONS

Attenuated Humoral Response to Seasonal and Pandemic (A/H1N1 2009) Virosomal and MF-59 Adjuvated Influenza Vaccines in Complete Remission Non-hodgkin Lymphoma Patients Previously Treated With Rituximab Containing Regimens

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Influenza vaccination is generally recommended in Lymphoma patients, but patients treated with rituximab (anti-CD20 mAb) seem to be unable to mount an adequate humoral response to naïve/recall antigens during the peri-treatment time. Here, we analyzed the humoral response (hemagglutinin inhibition assay) to seasonal influenza vaccination in two consecutive influenza seasons (2008/2009 and 2009/2010) as well as the response to naïve influenza antigen (pandemic H1N1/2009) during season 2009/2010 in complete remission NHL for at least 6 months after the last rituximab administration. An intramuscular virosomal inactivated vaccine was used during the season 2008/2009 (RIT-01 study) and intramuscular MF-59 adjuvated vaccines were used during the seasons 2009/2010 (RIT-02 study. The CHMP/EMEA immunogenicity criteria were assessed: seroconversion rate (SC), seroprotection rate (SP), and mean fold increase of antibody titer were determined. Median time after rituximab administration was 29 months in the RIT-01 study and 33 months in the RIT-02 study. In the RIT-01 study, the response to one dose of seasonal vaccine in 31 NHL patients was compared to that of 34 age matched healthy volunteers (HV). In the RIT-02 study, the response to pandemic vaccine (two doses) followed by single-shot seasonal influenza vaccine in 14 NHL patients was compared to that of two cohorts of 14 age-matched HV vaccinated with the same seasonal or pandemic vaccination schedule. In both the studies patients had a strongly attenuated but not completely suppressed response to seasonal and pandemic influenza vaccine. According to the CHMP/EMEA criteria patients in the RIT-01 study do not appear sufficiently protected. Patient response to pandemic vaccine (RIT-02 study) was weak but it was boosted by the second dose (reaching levels similar to those observed in HV after one dose). Fifty-five % of patients enrolled in the RIT-01 study and 43% of patients enrolled in the RIT-02 had either IgG, IgA or IgM serum level below the normal rage. CD27 + memory B-cell populations were significantly depleted in the patients in both the studies ($P < 0.001$). In conclusion, patients treated with rituximab-congaing regimens have a significant lack of humoral response to influenza vaccine compared with healthy controls, even long time after treatment administration, associated with depletion of CD27 + memory B cells and hypogammaglobulinaemia. Nevertheless, influenza vaccination should be still recommended/offered in this setting.

Key Words: B cell, Lymphoma, Immunotherapy.

The Proclaim^sm (Proleukin® Observational Study to Evaluate Treatment Patterns and Clinical Response in Malignancy) Study: The Response Rates for High Dose Interleukin-2 (HD IL-2) Therapy

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The primary aim of the PROCLAIM^SM Registry is to establish a standardized source of observational data that can be used to report and query patient care patterns, clinical outcomes and trends from HD IL-2 therapy in treating metastatic melanoma (MM), renal cell carcinoma (RCC). As part of the registry retrospective data were collected from 268 patients. Consecutive patients were entered at each of the 13 participating sites.

The primary aim of the PROCLAIM Registry is to establish a standardized source of observational data that can be used to report and query patient care patterns, clinical outcomes and trends from HD IL-2 therapy in treating metastatic melanoma (MM), renal cell carcinoma (RCC). As part of the registry retrospective data were collected from 268 patients. Consecutive patients were entered at each of the 13 participating sites.

Key Words: Interleukin-2, Immunotherapy.

Demographics	
Age (mean ± SD)	53.2 y ± 11.5
Gender	178 M, 90 F
Diagnosis	169 MM, 99 RCC
Number of metastatic sites (median, min, max)	2 (1,6) (n = 248)
ECOG Status at baseline (n = 255)	0-74%; 1-24%; 2-1%
LDH at baseline (median, min, max)	178 (74,1282) IU/L (n = 139)

Physician Reported Response Assessment (N = 243)

	Melanoma% (n)	Renal Cell% (n)
Complete Response	1 (2)	2 (2)
Partial Response	16 (23)	18 (17)
Stable Disease	28 (41)	35 (33)
Progressive Disease	56 (88)	45 (43)
Total	148	95

PD-L1 Blockade Improves the Efficacy of Adoptively Transferred Tumor Infiltrating Lymphocytes in a Colon Carcinoma Model

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Colon cancer is the fourth most common cause of cancer death worldwide, with 141,000 new cases diagnosed in the United States in 2011. The presence of tumor-infiltrating lymphocytes (TIL) has been associated with improved survival in patients with colon cancer. Within the tumor environment, multiple factors, including regulatory T cells, myeloid derived suppressor cells, and expression of co-inhibitory signals such as CTLA-4, PD-1 and PD-L1, can lead to the inactivation of TIL. Hence, there is a need to develop strategies that disrupt these negative regulators in the tumor microenvironment in order to achieve robust anti-tumor immune responses. In this study, we treated mice bearing the MC-38 colon carcinoma with anti-PD-L1 antibodies and evaluated the reactivity of TIL. In vitro expanded TIL from tumor-bearing mice treated with anti-PD-L1 antibodies demonstrated a significant increase in cytotoxic T cell responses (28% lysis) compared to TIL from mice treated with normal rat IgG (rIgG) antibodies (17% lysis, $P < 0.01$). We measured increased numbers of CD4 + and CD8 + T cells infiltrating the tumors of PD-L1 antibody-treated mice compared to mice treated with rIgG antibodies ($P < 0.01$). Finally, adoptive transfer of in vitro expanded TIL purified from the tumors of PD-L1 antibody-treated mice in combination with dendritic cell vaccination led to a delay in MC-38 tumor growth ($55\,mm^2$) compared to mice that received TIL from rIgG-treated mice ($100\,mm^2$, $P < 0.05$). These findings suggest that blockade of PD-L1 increases T cell infiltration into tumors and adoptive transfer of these T cells enhances anti-tumor immunity. This strategy may lead to improvements in the treatment of patients with colon carcinoma.

Key Words: Colorectal cancer, Combination immunotherapy, PD-1.

Haploidentical Natural Killer Cells Plus Monoclonal Antibody 3F8 for Resistant High-Risk Neuroblastoma: Preliminary Results of an Ongoing Phase I Study

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Background: Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) is a potent mechanism of 3F8 activity against neuroblastoma (NB). KIR and HLA genotypes define NK activity and are key prognostic markers in 3F8-treated patients. NK-cells are depleted by standard NB chemotherapy, but when rescued by allogeneic NK transfusions, can be optimized for anti-NB cytotoxicity by selecting NK donors for maximum NK ADCC: from either licensed NK-cells responding to "missing self" or from unlicensed NK-cells responding to "missing ligand".

Methods: We initiated a phase I study of the combination of haploidentical NK-cells and anti-GD2 antibody 3F8 for the treatment of refractory or recurrent high-risk NB (http://www.clinicaltrials.gov NCT00877110). The primary objective was to determine the maximum tolerated NK-cell dose (MTD). Secondary objectives included assessing anti-NB activity and its relationship to KIR/HLA genotypes, NK function, and NK chimerism. Eligibility criteria included availability of $\geq 2 \times 10^6$ CD34 + autologous cells/kg. Patients received a lymphodepleting regimen of high-dose cyclophosphamide, topotecan and vincristine (days 1-3) prior to infusion (day 5) of NK-cells isolated from donor leukophereses using a process of CD3-depletion (to $< 2 \times 10^4$ CD3 + cells/kg) followed by CD56-enrichment. 3F8 ($20\,mg/m^2/d$) was administered on days 8-12. NK-cell dose-escalation occurred in the absence of dose-limiting toxicity (DLT). Cytoreduction-related side effects were not considered DLT.

Results: Ten patients have been treated thus far: 8 at dose-level 1 (1-4.99×106 CD56 + cells/kg) and 2 at dose-level 2 ($5\text{-}9.99 \times 10^6$ CD56 + cells/kg). MTD has not yet been reached. One patient at dose level 1 developed DLT: grade 3 vomiting and hypertension. No other > grade 2 unexpected therapy-related toxicities were encountered. Neither GvHD nor myeloablation requiring stem cell rescue was observed. 2 patients achieved complete response.

Conclusion: Preliminary results from this first-in-pediatrics trial of NK-cells plus antibody against solid tumors suggest that the combination is safe following cytoreduction and may be effective for some patients with high-risk NB.

Key Words: Combination immunotherapy, Neuroblastoma, NK cells.

Pharmacological Characteristics of Peperomia Pellucida (L.) Kunth as Well as Ficus Pumila L. and its Application in Immunological Cells of Wistar Rats

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Plants are vital for existence of life on earth. The plants around the habitats of the world population not only provide food for living organisms, but also produce different chemicals necessary for human health. Extracts of some plants have been reported to play a contributory role in enhancing immune function. The studies were evaluated and compared the effects of single and combined oral administration of fresh aqueous Peperomia pellucida (L.) Kunth, and Ficus pumila L. extracts at different concentrations on some immunological determinants in Wistar rats. Cluster of differentiation 4 (CD4) cells of the Wistar rats were estimated using Partec flow cytometric technique, while total and differential white blood cell (WBC) counts were estimated using the automated haematology analyzing technique. The studies revealed that, CD4 cells and total WBC counts were significantly increased ($P \leq 0.05$) in a dose-dependent manner in both Peperomia pellucida (L.) Kunth (250 mg/Kg/d:350 ± 12 cell/ul and $2.75 \pm 0.15 \times 10^3$ cell/l, 500 mg/Kg/d:390 ± 11 cells/μl and $3.05 \pm 0.10 \times 10^3$ cell/l, 750 mg/Kg/d:600 ± 10 cell/μl and $3.25 \pm 0.05 \times 10^3$ cells/l); and Ficus pumila L. (250 mg/Kg/d:450 ± 12 cell/ul and $2.85 \pm 0.15 \times 10^3$ cell/l, 500 mg/Kg/d:495 ± 33 cells/μl and $3.30 \pm 0.10 \times 10^3$ cell/l, 750 mg/Kg/d:685 ± 10 cell/μl and $3.55 \pm 0.05 \times 10^3$ cells/l) treated Wistar rats when compared to the zero control (200 ± 10 cells/μl and $1.55 \pm 0.05 \times 10^3$ cells/l, respectively). Extract of Ficus pumila L. at 750 mg/Kg/d had significantly increased the CD4 cells and total white blood cell count when compared to other concentrations ($P \leq 0.05$). But no significant effect was observed on these parameters when extracts were combined (250 mg/Kg/d:259 ± 24 cell/μl and $1.85 \pm 0.15 \times 10^3$ cells/l, 500 mg/Kg/d:325 ± 21 cells/ul and $2.15 \pm 0.10 \times 10^3$ cells/l, 750 mg/Kg/d:369 ± 10 cells/μl and $2.35 \pm 0.05 \times 10^3$ cells/l respectively), the differential WBC count showed a significant increase in the proportion of cell types (lymphocytes, neutophils, and monocytes) $P \leq 0.05$. The outcomes from the studies revealed the immune boosting capabilities of Peperomia pellucida (L.) Kunth, and Ficus pumila L., but underscored their synergistic activities. Proper scientific studies conducted on the

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above plants may lead to discovery of more effective drugs than in use at present.
Key Words: Animal model, CD4+ T cells, Combination immunotherapy.

Aging Results in Acute TNF-α-dependent Lethality Following Systemic Immunotherapy: Impact of Body Fat Content

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Aging is associated with an increased distribution of visceral fat and loss of lean body mass. Emerging data has recently associated obesity with increased induction of proinflammatory cytokines and immune activation. Specifically, adipocytes have been shown to release adipokines, such as TNFα, which can directly induce T cell and macrophage responses towards chronic inflammatory states. Conversely, caloric restriction has been shown to enhance metabolic efficiency, increase life-span, and increase resistance to oxidative stress. Previous studies comparing aged and young mice treated with agonistic αCD40/IL-2 immunotherapy (IT) resulted in aged mice undergoing rapid lethality within two days of treatment as opposed to young mice, whom are able to tolerate IT treatment. Aged mice exhibited lethal toxicities via increased pathological levels of serum proinflammatory cytokines (TNFα, IL-6, IFNγ), and increased multi-organ damage to the liver, lung and gut. Given recent published evidence that fat plays a role in inflammation, we sought to determine if fat contributes to immunotherapy induced toxicities. We assessed the impact of IT treatment on young obese (ob/ob) mice in comparison to aged caloric-restricted (CR) and age-matched control (WT) mice. Young ob/ob mice showed greater serum levels of TNFα, IL-6 and IFNγ after IT, similar to aged mice, in comparison to age-matched controls. Aged-CR mice had reduced proinflammatory cytokine levels and displayed less pathology in response to IT compared to aged ad libitum fed mice. In vivo depletion of macrophages in aged mice resulted in lesser cytokine levels and protection from pathology. Both TNF-α-deficient mice and in vivo TNF blockade in tumor-bearing aged mice resulted in increased survival due to protection from toxicity and anti-tumor effects. The data demonstrate an intricate relationship between TNFα, macrophages and body fat as factors in the age-associated pathologic responses to systemic IT.
Key Words: Toxicity, Combination immunotherapy, Macrophages.

HER-2 Peptide Vaccination Suppresses Spontaneous Tumorigenesis and Tumor Stem Cell Expansion in MMTV-PYVT Transgenic Mouse Model

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Immunization targeting HER-2 could have considerable therapeutic potential by controlling growth and metastasis of highly aggressive tumor cells in the earlier preclinical and clinical studies. Just a few studies have examined preventive potential of HER -2 vaccines in preclinical studies. However, animal model systems used in the previous studies were tumor transplantation or neu-transgenic mouse, which were not relevant to human HER-2 positive breast tumorigenesis. In this study, active immunotherapy against tumor antigen HER -2/neu for primary prevention of breast cancer was tested using FVB/N-Tg (MMTV-PyVT) transgenic mice model. Mice were grouped to receive either HER -2 peptide vaccine, immune adjuvant only, tetanus toxoid, or PBS every 2 weeks for 3 times and monthly thereafter. The MMTV-PyVT transgenic mice in control groups (PBS, immune adjuvant only, or tetanus toxoid peptide) developed spontaneous mammary adenocarcinomas in 12 to 15 weeks, but vaccination against HER-2 strongly suppressed tumor formation by 30 weeks of observation. Further pathologic examination showed complete prevention of tumorigenesis was observed in HER-2 vaccinated mice, whereas the mice in control groups developed highly aggressive HER-2 over-expressing tumors similar to human breast cancer. The tumor protective effect of peptide vaccination was associated with induction of HER-2-specific humoral immune responses as well as T cell responses. Tumors from HER-2 peptide vaccine group showed a significantly higher level of CD8 T cell infiltration. Additionally, role of signal through HER-2 pathway and the relationship with stemness of cancer cells were determined by Aldefluor assay, mammosphere formation assay using Mouse mammary carcinoma (MMC) cells in vitro. Further analysis of mammosphere formation capacity of MMC cells using immune sera showed that sera from HER-2 vaccinated mice had a significant inhibitory effect on mammosphere formation in HER-2 overexpressing MMC cells. These results suggest that HER-2 targeting by cancer vaccination might be useful adjuvant to standard therapy, helping to prevent relapse in patients with HER-2-over-expressing tumors by suppressing stem/progenitor cell population.
Key Words: Breast cancer, Antibody response, Active immunotherapy.

Topical Imiquimod Induces Immune Activation and Regressions of Cutaneous Melanoma Metastases

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Introduction: Topical imiquimod, a toll-like-receptor 7 agonist, may induce regression of some cutaneous melanoma. However, its role and potential mechanism of effect have not been addressed systematically in metatases. Toward this end, we examined two hypotheses: 1) Topical imiquimod monotherapy may induce regressions of intransit metastases of melanoma; 2) Combination immunotherapy of topical imiquimod with cutaneous peptide vaccination may induce immune signatures in the tumor microenvironment.
Methods: Historical evaluation of imiquimod monotherapy: From 2005-2009, 9 evaluable patients were identified from our melanoma database with cutaneous metastases-in-transit treated with imiquimod. Clinical outcomes were recorded. Kaplan-Meier curves were generated with IBM SPSS v.20, and significance evaluated by the log rank test. Assessing intratumoral effects of topical imiquimod combined with cutaneous peptide vaccination: 14 patients are being enrolled in a pilot study of topical imiquimod plus multi-peptide melanoma vaccine (NCT01264731). Initial data are available from the first 3 patients, who underwent tumor biopsies pre-treatment (d1), after 3 vaccines + 21 days topical imiquimod (d22) and after a total of 4 vaccines and 42 days imiquimod (d43). 12 biopsies have been obtained: 3 d1, 5 d22 (3 treated, 2 untreated), and 4 d43 (3 treated, 1 untreated). RNA was isolated from each specimen, and microarray analysis performed with Partek Genomics Suite and Ingenuity Pathway Analysis software using probe intensity data derived from the Affymetrix Human Gene 1.0 ST Array.
Results: Of 9 patients treated with topical imiquimod monotherapy from 2005-2009, 2 (22%) had complete cutaneous responses, 3 (33%) had mixed response of different lesions and 4 (44%) had progression of all lesions. Gene expression analysis, with unchaperoned clustering at $P < 0.005$, yielded a distinct separation between treated and untreated tumors, with similar changes on day 22 and day 43. Functional gene analysis of treated tumors (day 22 + day 43) compared to day 1 at $P < 0.01$ overwhelmingly

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revealed upregulated immune signatures in treated tumors, with highly significant increases in canonical pathways including T-cell receptor signaling, TLR signaling, interferon signaling and dendritic cell maturation.

Conclusion: Topical imiquimod for cutaneous metastases of melanoma can induce control of skin metastases of melanoma, and induces immunologic signatures in the tumor microenvironment. Imiquimod has promise for treatment of melanoma metastases as part of combination immunotherapy.

Key Words: Advanced cancer immune response, Combination immunotherapy, Tumor microenvironment.

Blocking IL-18 During IL-12 + IL-15 Therapy Ameliorates Toxicity and Eradicates Malignancy

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IL-12 induces tumor regression through the activation of T cells, but is limited as an anti-tumor therapeutic because IL-12 is prone to cause rapid tachyphylaxis. IL-15 is a promising cytokine to help overcome IL-12 induced tachyphylaxis because it induces NK cell activation and proliferation, protects NK and T cells from apoptosis and maintains CD8 memory T cells. However, IL-15 in combination with IL-12 caused significant toxicity in mice limiting its usefulness as a therapy. Because IL-15 induces IL-18, which also causes severe toxicity in combination with IL-12, we investigated whether much of the IL-15 induced toxicity could be ascribed to IL-18 and whether IL-18 blockade could alleviate toxicity while maintaining antitumor activity when given with IL-15 + IL-12. To block IL-18 we hydrodynamically delivered a plasmid encoding the naturally occurring IL-18 binding protein (IL-18bpa), which caused sustained serum levels of IL-18bpa out to 21 days. Using an escalating dose of IL-15 in combination with IL-12, we examined the ability of IL-18bpa to alleviate the toxicity initiated by IL-15 + IL-12 treatment of mice. Treatment with IL-18bpa was able to block mouse morbidity at all doses tested, while vector control-treated mice became moribund at higher doses of IL-15. Additionally, IL-18bpa prevented the IL-15-induced increase in blood and peritoneal neutrophils often associated with toxicity, while not affecting the increase in beneficial NK and CD8 T cells. Next, we examined the anti-tumor potential of IL-18 blockade in combination with IL-15 + IL-12. The anti-tumor activity of IL-15 + IL-12 therapy against mouse renal cancer (Renca) growing in the peritoneal cavity was actually enhanced in the presence of IL-18bpa as compared to those mice that did not receive IL-18bpa. Thus IL-18bpa enhances the anti-tumor therapeutic potential of IL-15 + IL-12 therapy and alleviates treatment toxicity. These studies support the notion that treatment with IL-18bpa might offer clinical applications in combination cytokine therapies that are toxic due to induction of IL-18.

Key Words: IL-12, Interleukin-15, Toxicity.

Utilizing Effector B Cells in Cancer Adoptive Immunotherapy

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We hypothesize that successful anti-cancer treatment will, in the final analysis, have to appropriately stimulate both humoral as well as cellular immunity. To this end, we reported that simultaneous targeting of CD3 on T cells and CD40 on B cells augmented the antitumor reactivity of tumor-primed LN cells. These studies established a role for engaging CD40 on tumor-draining lymph node (TDLN) B cells in the generation of effector cells. We also reported that IL-21 augmented the efficacy of T cell therapy by eliciting concurrent cellular and humoral responses. This study confirmed an interactive role between tumor-specific humoral responses related to IL-21 administration and adoptively transferred effector T cells. In recent study, we identified TDLN B cells as effector cells in an adoptive immunotherapy model. In vivo primed and in vitro activated TDLN B cells alone mediated effective ($P < 0.05$) tumor regression after adoptive transfer into two histologically distinct murine tumor models. B cell plus T cell transfers resulted in substantially more efficient antitumor responses than B cells or T cells alone ($P < 0.05$). Activated TDLN B cells produced IgM, IgG and IgG2b, which bound specifically to tumor cells and led to specific tumor cell lysis in the presence of complement. In a third tumor model, the 4T1 breast cancer spontaneous metastases model, we found that adoptive transfer of activated 4T1 TDLN B cells alone mediated significant inhibition of spontaneous metastases of the 4T1 cells from the injection site (the mammary fat pad) to the lung. Examination of the host revealed that the adoptive transfer of these B cells resulted in the induction of tumor specific T cell immunity as measured by cytotoxicity and cytokine (IFNγ and GM-CSF) production. Importantly, we found that the 4T1 TDLN effector B cells could directly and specifically kill the 4T1 tumor cells in the absence of antibody. More mechanistic studies revealed that adoptively transferred IL-10-/- TDLN B cells mediated significantly more effective antitumor immunity than equal numbers of WT TDLN B cells ($P < 0.05$). Adoptively transferred IL-10-/- 4T1TDLN B cells increased the production of IgG which bound to 4T1 tumor cells and significantly increased CTL activity mediated by host B cells as well as T cells in an immunologically specific fashion. While the role played by B cells in the host immune response to cancer is complex and controversial, our results indicate that in vivo primed and in vitro activated TDLN B cells can function as effector cells in cancer adoptive immunotherapy, and removal of IL-10-producing B cell subsets may represent an effective strategy to augment the therapeutic efficacy of the TDLN effector B cells.

Key Words: Adoptive immunotherapy, B cell.

Phenotypic and Functional Parallels Between Antigen-non-specific CD8 + Memory T Cells Following Cancer Immunotherapy and Influenza Infection

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One of the hallmarks of the adaptive immune system is the development of specificity towards antigen by immune cells. Memory T cells are an important component of acquired immunity, providing the host a great deal of protection from infectious agents due to their antigen specificity, rapid acquisition of effector function and low requirement for co-stimulation. Acute viral infection leads to activation and proliferation of antigen-specific CD8 + T cells, which play a critical role in viral clearance. In addition to the expansion of antigen-specific T cells, the presence of bystander activated CD8 + T cells has been well documented in various pathogenic infections, autoimmunity, and immunotherapeutic regimens for cancer. The phenotypic differences between antigen-specific versus antigen-nonspecific memory CD8 + T cells are consistent with TCR dependent (anti-CD3/anti-CD28) or independent (cytokine based) conditions, respectively. In previous studies, we have shown that anti-tumor immunotherapy with agonistic anti-CD40/IL-2 induced marked expansion of memory CD8 + T cells displaying increased expression of NKG2D but not of CD25 on the CD44highCD8 + T cells, indicative of bystander activation. Likewise, infection of naïve mice with two different strains of influenza resulted in rapid expansion of memory CD8 + T cells with this same bystander phenotype. Immunotherapy-based stimulation of memory CD8 + T cells also resulted in antigen-nonspecific proliferation and activation in the absence of TCR ligation, with NKG2D ligation inducing MHC-unrestricted cytotoxicity and leading to anti-tumor effects. Furthermore, NKG2D blockade resulted in increased tumor volume in immunotherapy

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treated mice bearing subcutaneous Renca tumors. Likewise, influenza infection with the concurrent administration of intranasal NKG2D blockade resulted in a significant increase in viral replication during early stages of infection. These anti-tumor and antiviral effects may be attributed to the upregulation of various NKG2D ligands on tumors and virally infected cells and may predispose these cells to control by bystander CD8 + T cells via primary effector mechanisms. Thus, our data suggest a common mechanism whereby antigen-nonspecific CD8 + T cells play an important, NKG2D-mediated, role in both anti-cancer immune responses and viral clearance.

Key Words: Memory CD8 + T cells.

Regulatory T Cells and Myeloid-derived Suppressor Cells in the Tumor Microenvironment Undergo FAS-dependent Cell Death During IL-2/ACD40 Therapy

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The presence of immunoregulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) within the tumor microenvironment represent major obstacles for cancer immunotherapy. A combination strategy which couples activation of anti-tumor immune responses with the removal of Tregs and MDSC should increase the durability of anti-tumor responses. Previously, we showed that systemic administration of Interleukin (IL)-2 and agonistic CD40 antibody (aCD40) elicited synergistic anti-tumor responses. This process was associated with the infiltration of tumors by effector CD8 + T cells, M1 macrophages and NK cells concomitant with the dramatic removal of Tregs and MDSC. In this study, we demonstrate that the loss of Tregs and MDSC from the tumor microenvironment after IL-2/aCD40 occurs through a Fas-dependent pathway. Among tumor-infiltrating leukocytes, CD8 + T cells, neutrophils and immature myeloid cells expressed Fas ligand. Furthermore, Fas was expressed by tumor-associated Tregs and immature myeloid cells, including MDSC. Tregs and MDSC in the tumor microenvironment also expressed activated caspases following IL-2/aCD40 treatment. In contrast to effector T cells, Tregs significantly downregulated Bcl-2 expression after IL-2/aCD40 therapy. Tregs and MDSC in the spleen also expressed activated caspases, however this was overcompensated for by the dramatic proliferation of these cells, as evidenced by BrDU uptake by both splenic Tregs and MDSC. Finally, the adoptive transfer of Fas-deficient Tregs into wildtype, Treg-depleted hosts, resulted in the persistence of a Treg population in the tumor microenvironment which was resistant to the IL-2/aCD40 and Fas-mediated apoptosis. The efficacy of IL-2/aCD40 was abrogated in these recipient mice, demonstrating the critical importance of Fas-mediated Treg removal towards the anti-tumor efficacy of this combination therapy. Our results suggest that immunotherapeutic strategies, such as IL-2/aCD40, that target the susceptibility of Tregs and MDSC to Fas-mediated cell death hold promise for further development as cancer treatment strategies.

Key Words: Combination immunotherapy, Treg cells, Tumor microenvironment.

Immunogenicity of P53 Synthetic Long Peptide Vaccination With Interferon-α Results in Increased P53-specific Polyfunctional T-cell Frequencies

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We previously established safety and immunogenicity of our vaccine, consisting of p53 synthetic long peptides (p53-SLP®) emulsified in Montanide ISA-51 VG. Upon 2 vaccinations p53-specific CD4 + T-cell responses were induced in 9 out of 10 colorectal cancer patients as measured by IFNγ-Elispot and proliferation. However, these T cells were not optimally polarized. In the current trail we investigated whether combination of Interferon-alpha (IFN-α) with p53-SLP® is both safe and able to improve the induced p53-specific IFN-γ response. Eleven colorectal cancer patients successfully treated for metastatic disease were enrolled in this study. Nine patients completed follow up after 2 injections with p53-SLP® together with IFN-α. Safety and p53-specific immune responses were determined before and after vaccination.

Cryopreserved PBMCs of the current trial were head-to-head compared to those obtained in our previous trial (p53-SLP® only). Of note, there were no overt clinicopathological differences between the 2 patient groups.

Toxicity of p53-SLP® vaccination combined with IFN-α was limited to grade 1 or 2, with predominantly small ongoing swellings at the vaccination site. Most patients (6/7) showed p53-specific IgG antibodies, while this humoral response was less broadly observed in patients with p53-SLP® only, which indicates an overall better p53-specific T-helper response. Addition of IFN-α to p53-SLP® vaccination also significantly improved the frequency of p53-specific T cells in IFN-γ ELISPOT ($P = 0.02$). Moreover, in the current trial, p53-specific T cells were detectable in blood samples of all patients in a direct ex vivo multiparameter flowcytometric assay (in which cells were stained for CD3, CD4, CD8, CD154, CD137, IFNγ and IL-2), opposed to only 2 out of 10 patients vaccinated with p53-SLP® only. Finally, the migratory capacity of the circulating p53-specific T cells was assessed by their presence in a biopsy of the second vaccination site. In the current trial all 5 successfully cultured skin biopsies harbored p53-specific T cells, while this was only the case in 2 out of 4 biopsies from the previous trial.

In conclusion, our study shows that p53-SLP® vaccination combined with IFN-α injection is safe and capable of inducing p53-specific immunity. When compared to a similar trial with p53-SLP® vaccination alone the combination was found to induce significantly more IFN-γ producing p53-specific T-cells.

Key Words: Colorectal cancer, Cancer immunotherapy, Cellular immunity.

The Transient Nature of Significant Toxicities Associated With High Dose Interleukin (HD IL-2): Preliminary Data From The Proclaim℠ Study

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Treatment with HD IL-2 has long been associated with life-threatening adverse events such as capillary-leak syndrome. However, minimal data exists detailing the duration of these events. The PROCLAIM℠ registry was established to generate a database from which the use of HD IL-2 may be better understood and refined. Data from a cohort of 268 patients from 13 sites in the US, with either metastatic melanoma (n = 169) or renal cell carcinoma (n = 99), has been entered. Each site retrospectively entered consecutive patients. Two sites administered HD IL-2 in their ICU; all other sites treated patients in either a step-down unit or on the oncology floor. All patients received at least one cycle of HD IL-2, 249 two cycles, 87 three cycles, 80 four cycles, 28 five cycles and 26

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received six or more cycles. The most frequent dose-limiting toxicities for cycles 1 and 2 were hypotension (29%, 43%), renal toxicity (30%, 41%), thrombocytopenia (26%, 17%), neurologic disorders (19%, 18%) and GI disorders (16%, 18%). In cycle 1, 29% pts required pressor support, this number increasing to 43% by cycle 2. Colitis, hepatitis, autoimmune phenomena such as acute or persistent arthralgia or endocrinopathy other than hypothyroidism were not observed. No infections or deaths were reported in this cohort. Importantly, values for blood and biochemistry variables universally returned to pre-dose values prior to the first dose of the next cycle, usually within 10 days. This demonstrates the transient nature of these toxicities and suggests the potential to explore HD IL-2 in combination or sequence with other therapy.

Key Words: Interleukin-2, Immunotherapy.

	Serum Creatinine (mg/dL)	Bilirubin (mg/dL)	Platelet Count (×109/L)
Pre-Cycle 1			
Mean	1.01	0.61	243
SD	0.31	0.51	81
N	251	247	253
Nadir or Peak Cycle 1			
Mean	2.27	3.32	84.6
SD	1.62	2.08	54
N	243	232	210
Pre-Cycle 2			
Mean	1.02	0.84	366
SD	0.33	0.50	157
N	228	229	229

Forced Light Expression in Tramp Tumors Induces Prostate Cancer Specific Immunity and Reduces Tumor Volume

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The forced expression of LIGHT molecules (a ligand for the lymphotoxin beta receptor) in an HPV induced cervical cancer tumor model has been shown in our lab to cause the recruitment of naïve T cells into the tumor-microenvironment and induce HPV-specific immunity leading to increased survival. Forced LIGHT expression has not been studied in a prostate cancer setting where tolerance to self-antigens exists. Here we tested the hypothesis that forced expression of LIGHT in prostate tumors would result in tumor regression by inducing prostate cancer-specific immunity and/or inhibiting the suppressive activity of Tregs. Quantitative real-time PCR and flow cytometric data showed that prostate cancer TRAMP-C2 cells can successfully express LIGHT molecules via infection with an adenovirus vector in vitro. In addition, intratumoral injections of LIGHT into TRAMP-C2 tumors in vivo caused an increase in tumor infiltrating lymphocytes and a decrease in Treg cells. The suppressive function of these Tregs was also compromised as shown by their decreased release of suppressive cytokines and their decreased suppressive capacity towards effector cells. In addition our data showed that the intra-tumoral delivery of LIGHT encoding adenoviral vectors into TRAMP-C2 tumor bearing mice can delay tumor growth while inducing prostate stem cell antigen specific IFN-gamma releasing CD8+ T cells. In conclusion, treatment of prostate cancer through forced LIGHT expression induces prostate cancer specific immunity, reduces tumor induced immune suppression and results in an increased survival (Figs. 4, 5).

Key Words: Treg cells, Tumor associated antigen, Tumor infiltration lymphocytes.

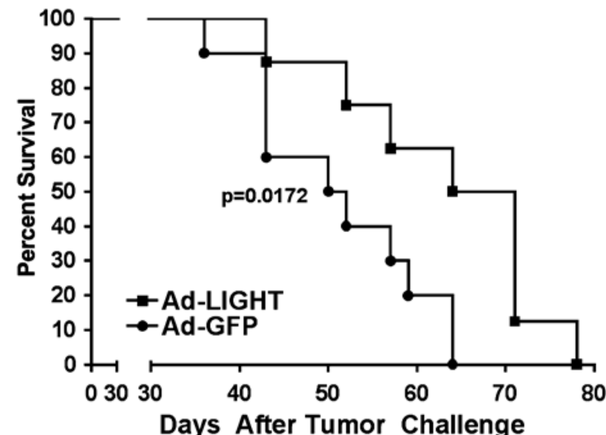

FIGURE 4. LIGHT treatment delays prostate tumor growth and increases survival time.

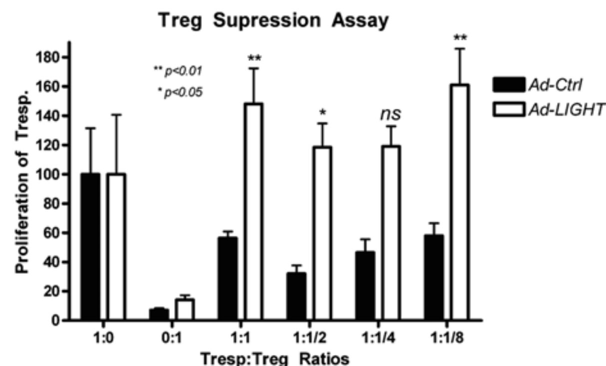

FIGURE 5. Regulatory T cell suppression is lost subsequent to Ad-LIGHT treatment.

INNATE IMMUNITY IN CANCER

NK Cell Molecular Signatures are Predictive of Relapse Free Survival of Favorable Prognosis of Breast Cancer Patients

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An "immune response" signature has been associated with improved outcomes in several tumor types. Particularly, such immune signature has been linked to increased activity of adaptive immune effector cells recognizing antigens expressed by tumor cells. However, experimental as well as clinical observations suggest that immune-mediated tissue destruction is dependent upon coordinate activation of immune effector genes expressed by cells of the innate and adaptive immune systems. Among cells of the innate immune system, natural killer (NK) cells can play a major role in resisting tumor progression. Recognition of tumor cells by NK cells is mediated by the interaction of activating receptors with ligands expressed on the tumor target cells which fail to express ligands specific for NK cell inhibitory receptors. NK cells also express adhesion molecules, thereby interacting with tumor cells and DCs mediating their disruption or activation, respectively. Here, we investigated whether the coordinate expression of NK activating

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receptors and adhesion molecules could provide a signature to segregate breast cancer patients into relapse and relapse-free outcomes. Gene expression profiling, RT-PCR screening and survival analysis were performed on RNA extracted from primary breast cancers. Tumors were obtained from patients experiencing either 5-9 years relapse-free survival or tumor relapse within 1-6 years following initial treatment. Based on differential expression of CD56 and CD16, NK cells did not vary between relapse free and progressing groups.However, tumors from patients with no recurrence were characterized by up-regulation of Natural Cytotoxicity Receptors (NCRs), NKG2D, CD226 (DNAM-1) and CD96.. Indeed, expression of NK cells inhibitory receptors transcripts including KIR2DL3 and KIR3DL3 was not significantly different in patients with widely diverging outcomes and their expression was negatively correlated with NCRs expression. This confirmed previous observations suggesting that the balance between inhibitory and activating pathways in NK cells are finely regulated at the transcriptional level and activating pathways are central modulators of NK function in the tumor microenvironment.The NK cells parameters identified in this study, together with the prognostic B and T cell signatures previously reported by us, highlight the effector cooperation between the innate and adaptive immune components within the tumor microenvironment and represent a powerful tool for predicting breast cancer outcome which might be easily introduced in clinical practice.

Key Words: Micrenvironment, Tumor immunity, Innate immunity.

Resolution of Incoherent Stimuli During Macrophage Polarization

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Macrophages play a central role in balancing protective inflammation and homeostasis by functionally "polarizing" into either immunostimulatory (M1) or immunosuppressive (M2) phenotypes. Tumors promote macrophages polarization towards tumor-associated phenotypes (typically, M2-like), which support tumor survival. Notably, macrophages can also switch from M2 to M1 when exposed to certain stimuli, including interleukin-12 (IL-12). Although immunostimulatory and immunosuppressive stimuli rarely exist independently in vivo, most investigations into the regulation of polarization have applied coherent stimuli (either pro-M1 or pro-M2 stimuli alone). In order to investigate how macrophages respond to incoherent stimuli, we exposed macrophages to both IL-10 and IL-12 (pro-M2 and pro-M1 stimuli, respectively), followed by activation with lipopolysaccharide (LPS). Interestingly, the effects of IL-10 dominated those of IL-12, such that M2-type responses increased with IL-10 dose independently of IL-12 co-treatment. We also investigated competition between IL-10 and the canonical pro-M1 stimulus, IFNγ. Previous reports indicated that IFNγ pre-treatment suppresses IL-10's ability to inhibit LPS-induced TNFα production. In contrast, when we exposed cells to IL-10 and IFNγ simultaneously, IL-10 still inhibited LPS-induced expression of M1 genes including TNFα. Interestingly however, co-treatment with IFNγ blocked IL-10-induced expression of additional IL-10. This may suggest that even in tumor microenvironments with chronically elevated IL-10 levels, IFNγ could inhibit a feedback loop implicated in maintaining and promoting the polarization of macrophages into a tumor-supportive M2 phenotype.

We also hypothesized that incoherent stimuli might drive individual cells within a population to commit to different phenotypes. To investigate whether such heterogeneity exists, macrophages were treated with incoherent stimuli (IL-10 and IL-12), stained for uniquely M1 and M2 markers (intracellular TNFα and surface-expressed CD163, respectively), and profiled by flow cytometry. Under some conditions, distinct M1 and M2 cells were observed within a single population. The probability of polarization towards M2 increased with IL-10 dose and was independent of IL-12 co-treatment. Other cells remained non-responsive to LPS activation, and this probability of activation was unaffected by cytokine treatment. We also separated subpopulations by flow cytometry and confirmed that different subpopulations also exhibit distinct gene expression patterns, supporting the hypothesis that macrophage polarization may be stochastic and that separate regulatory mechanisms govern activation and polarization.

Key Words: Immunosuppression, Tumor immunity, Macrophages.

The AIM2 Inflammasome Inhibits IFN-β Production Triggered by Tumor-derived DNA

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Spontaneously T cell responses against a growing tumor mass frequently occur, despite the fact that most tumors lack an obvious infectious etiology. This observation raises the question of wich endogenous adjuvants provide activation signals for antigen presenting cells (APCs) that leads to productive T cell activation and differentiation. Our group has identified a critical role for host IFN-β production as a bridge to adaptive immunity against tumors. Recent work has indicated that tumor-derived DNA may be the ultimate ligand, which triggers a pathway involving the adapter STING to drive IFN-β production by APCs. Cytosolic DNA also can activate the AIM2 inflammasome, that leads to the activation of ASC and Caspase-1 and consequently the production of mature IL-1β. We therefore have studied a potential functional role of the AIM2 inflammasome in IFN-β production in response to tumor DNA. We observed that macrophages or DCs from mice deficient in AIM2, ASC or Caspase-1 produce markedly higher amounts of IFN-β after DNA stimulation. This observation suggests that the AIM2 inflammasome is inhibitory for the STING/IFN-β pathway. The mechanism of this inhibitory effect is being dissected. The possibility that inflammasome-deficient cells might be less susceptible to cell death was ruled out, as induction of apoptosis after tumor DNA loading was similar in WT and knockout macrophages. Another possibility is that AIM2 inflammasome could induce the release or activation of a soluble factor that might feedback on the cells and inhibit the STING pathway. However, there was no inhibition of IFN-β production when supernatants collected from IRF3-deficient macrophages was added to stimulated AIM2-/- or ASC-/- cells. Similar results were obtained when IRF3-/- and inflammasome-deficient macrophages were cocultured but separated by transwells. Our results indicate that the AIM2 inflammasome is inhibitory for the STING/IFN-β pathway in a cell-intrinsic mechanism. The crosslink between the STING and the AIM2 inflammasome pathways may have a regulatory effect in the immunological response against tumors which should be explored in vivo.

Key Words: Tumor immunity, Dendritic cell, Innate immunity.

Genomic Characterization of Melanoma Cell Lines

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Background: It is assumed that transcriptional signatures displaying a status of immune activation of cancer reflect genes expressed by infiltrating immune cells. However, it is possible that part of the immune signature is due to constitutive activation of cancer cells. We recently confirmed this observation by assessing the transcriptional profiles of 113 melanoma metastases according with the expression of Interferon regulatory factor (IRF1) with enhancement of Interferon IFNG type signature. In several cases, the in vivo activation observed in tissues corresponded to constitutive activation of cell lines in vitro. Thus, it is possible that the immune signatures expressed by cancer tissues are partially due to the activation of immune mechanisms intrinsic to the tumor cell

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biology. The present study focused on genomics characterization of melanoma cells lines with up regulated IFNG related genes and their relationship with immune activation in the parental tumors; in particular the relation between gene expression level, variation of copy number and mutational status of activated genes.

Methods: Transcriptional pattern, copy number variation and mutational status was correlated with constitutive levels of intracellular STATs and their relative activation (pSTAT) assessed by flow cytometry analysis. This information was correlated with the metastatic parental tissues.

Results: Class comparison (Student's *T*-test $P < 0.05$) identified more the 2000 genes differentially expressed by IRF1 positive compared to IRF1 negative cell lines. 154 genes IFNG related were found significantly differentially expressed between two groups (JAK1, JAK2, IFNGR1, IFNGR2, IL 15, IDO1). Also comparing the gene profiling data with the protein data we found that the samples with strong up-regulation of IFNG related genes have pSTAT1 constitutively activated. The analysis of copy number of the same genes identifies amplification for some of these genes. The constitutive activation of these transcriptional factors in vitro suggested that part of the profile observed in vivo could be related to the intrinsic biology of cancer cells rather than being limited to the activation of infiltrating immune cells.

Conclusion: This novel approach comparing genomic data with functional data for cell lines and their parental tissues may provide insights about the intrinsic genetic alterations of cancer cells driving the immune phenotype observed in some melanomas and other cancers. These preliminary observations support the notion that immune activation of some tumors is at least in part determined by the intrinsic biology of cancer cells.

Key Words: Tumor immunity, Immunomodulation, Melanoma.

Tumor Immune Subtypes Distinguish Tumor Subclasses With Clinical Implications in Breast Cancer Patients

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Introduction: Nowadays decisions regarding systemic therapy are largely based on classic prognostic and predictive factors, being nodal stage, tumor size, hormone and HER2 receptor status, which do not provide optimal risk-stratification. There is strong evidence that the host's immune response is able to control cancer progression and therefore has mayor prognostic and predictive implications. By determining markers of immune surveillance and immune evasion present in the tumor, we will define clinical applicable tumor immune subtypes in breast cancer and objectify the prognostic value hereof in a large cohort of breast cancer patients, with tailored systemic therapy in the near future being a primary focus of this study.

Patients and Methods: The study population (n = 677) consisted of all early stage breast cancer patients primarily treated with surgery in our center between 1985 and 1994. Sections of available formalin-fixed paraffin embedded tumor tissue (85%, 574/677) were immunohistochemically stained (IHC) for CD8 (CTL) and PEN5 (NK cells) to determine tumor infiltrating immune cells. Tumor expression of classical and non-classical HLA class I and tumor infiltrating Tregs (FoxP3) were also determined by IHC. Tumor immune subtypes were constructed based on underlying biology and quantification of the presence or absence of these markers.

Results: Patients with data available for all mentioned immune markers (59%, 336/574) were analyzed for tumor immune subtype determination. High, intermediate and low immune susceptible tumor immune subtypes were found in respectively 20% (68/336), 62% (208/336) and 18% (60/336) of patients. Tumor immune subtypes showed to be statistically significant prognostic factor for relapse free period (RFP) ($P < 0.001$, intermediate vs. high immune susceptible: hazard ratio (HR) 1.92, 95% confidence interval (CI) 1.14-3.24; low vs. high immune susceptible HR 4.00, 95% CI: 2.26–7.08) and relative survival (RS) ($P < 0.001$, low vs. high immune susceptible HR 4.58, 95% CI: 2.02–10.36; intermediate vs. high immune susceptible: HR 2.69, 95% CI: 1.24–5.81), independent of known clinicopathological parameters and with high discriminative power. A validation study is currently underway.

Conclusion: The breast cancer tumor immune subtypes that we define here represent a prognostic profile with solid underlying biological rationale and with high discriminative power. Tumor immune subtype profiling is a promising method for prognosis prediction and may aid in achieving tailored treatment for breast cancer patients.

Key Words: Breast cancer, Biomarker, Immune escape.

SIGLEC-7 and -9 Receptor-ligand Interactions in Human Cancer: A Potential Role For Tumor Escape From NK Cell Attack

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Altered surface glycosylation on malignant cells, especially sialic acid-containing carbohydrate determinants, may directly affect NK cell responses in tumor immunity and tumor editing events. Siglec-7 and -9 are MHC class I-independent inhibitory receptors on human NK cells. By virtue of their sialic-acid binding capacity they may participate in sialoglycan-mediated interactions during natural anti-tumor immune responses. In our work, we show that engagement of these receptors inhibits primary NK-cell mediated degranulation, IFNγ-secretion and cytolisis of target cells. In contrast to consistent surface expression of Siglec-7 on NK cells, Siglec-9 expression defines a subset of CD56dim NK cells with higher frequency in cord blood than in adult peripheral blood, which exhibits a more mature phenotype yet similar telomere length, and an enhanced expression of CXCR1 with concomitantly augmented chemotactic migration towards IL-8 as compared to Siglec-9- CD56dim NKs. The frequency of this Siglec-9 + CD56dim NK cell subset was significantly reduced in cancer patients. We report significant overexpression of Siglec-7 and -9 ligands on a broad variety of human tumor cell lines of different histological types and in tumor biopsies from melanoma patients. The enzymatic removal of Siglec-7 and -9 ligands on NK cell-susceptible tumor cells resulted in their increased killing both in vitro and in vivo in a mouse model with a reconstituted human NK-cell compartment. Altogether, our data highlight the potential of therapeutically targeting the Siglec-7 and -9, and their ligands, to efficiently enhance NK cell-mediated anti-tumor immunity.

Key Words: Tumor immunity, Immune escape, NK cells.

Intradermal Toll Like Receptor-2 (TLR2) Agonist Mycobacterium W (CADI-05) in the Treatment of BCG Refractory Non Muscle Invasive Transitional Cell Carcinoma of Bladder

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Background: TLR2 agonist, mycobacterium w (Cadi-05), a non-specific immunomodulator induces pure Th 1 response leading to tumor regression, following intradermal administration. It is well tolerated and has been found to enhance the efficacy of standard chemotherapeutic agents including but not limited to platinum compounds and taxanes (J. Clin. Onco 29:2011(suppl;abstr 7501)). This study was designed to evaluate the efficacy and toxicity of cadi-05 in patients (pts.) with BCG refractory non muscle invasive bladder cancer.

Methods: Twenty two patients with BCG refractory non muscle invasive bladder cancer received 0.1 mL Cadi-05 intradermally on each deltoid following TUR. This was followed by 0.1 mL Cadi-05 every fourteen days for 12 weeks, then every four weeks for 24 weeks and thereafter every eight weeks for 24 weeks. None of the patients received any intravesical or other systemic therapy. All were followed up with cystoscopy, cytology and biopsy at weeks 10,22,34,46 and 58.

Results: There were 11 patients each with Stages T1 and Ta at the start of treatment. At 22 weeks visit 11 patients (50%) were found to be disease free. Seven patients (out of these eleven patients) remained disease- free at the end of 58 weeks (study period). Of remaining patients with recurrence of disease prior to 22 weeks, disease recurred in less than 10 weeks in five. In none of the patients with recurrence was disease progression observed at time of recurrence. Systemic side effects seen include fever (5 patients, 8 episodes), body pain (2 patients,3 episodes), frequent urination (1 patient, 1 episode).

Conclusion: Intradermal Cadi-05 achieves and maintains complete remission in 32% beyond 58 weeks. Overall intradermal Cadi-05 treatment is fairly well tolerated.

Key Words: Genital tumors, Cancer immunotherapy, Innate immunity.

Recurrence Over Time

Visit	Week	Recurrence Free No. (%)
6	10	16 (77%)
9	22	11 (50%)
12	34	09 (41%)
14	46	07 (32%)
16	58	07 (32%)

TLR8 Agonist VTX-2337 Enhances Human NK Cell Function VIA Inflammasome Activation and IL-18 Induction

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The important role for NK cells in tumor immune surveillance is well established, and supported by reports of NK cell deficiencies in cancer patients. The current study was undertaken to assess the activity of VTX-2337, a potent and selective TLR8 agonist, on NK cell function. NK cells from 10 healthy donors were stimulated with VTX-2337 alone or VTX-2337 followed by subsequent CD16 or NKG2D crosslinking. VTX-2337 by itself induced IFN-γ production and CD107a degranulation in NK cells. Furthermore, pre-incubation with VTX-2337 significantly enhanced IFN-γ production and CD107a degranulation induced by CD16 and NKG2D crosslinking. The increase in percentages of IFN-γ + and CD107a + cells was observed in both CD56bright and CD56dim NK cells. The percentage of dual functional NK cells (IFN-γ + and CD107a +) was also significantly increased. MicroRNA-155, which has been recently shown to be an important regulator of IFN-γ production in NK cells, was significantly induced by VTX-2337. IL-18, a cytokine that is known to enhance NK cell function, was found to be induced by VTX-2337. While the induction of pro-IL-18 by TLR8 activation was expected, the formation of mature, secreted IL-18 is dependent on inflammasome activation. Therefore, we examined the potential effect of VTX-2337 on inflammasome. Results showed that NLRP3 inflammasome activation is involved in the effect of VTX-2337. Collectively, results from our study showed that TLR8 agonist VTX-2337 can enhance NK cell function (IFN-γ + production and CD107a degranulation) via NLRP3 inflammasome activation and induction of IL-18. Thus, VTX-2337 has the potential to enhance NK mediated anti-tumor activities in cancer patients.

Key Words: Immunomodulation, Innate immunity, NK cells.

IFN-GAMMA-inducible Protein-10 Stimulates Migration of Activated Natural Killer Cells Toward Melanoma Tumors

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Tumor infiltration by natural killer (NK) cells is associated with improved prognosis in patients with several different solid cancers. In mice, NK cell accumulation is dependent on production of IFN-gamma-inducible protein-10 (IP-10) by tumor cells. Here we investigated whether infusion of human NK cells would result in increased migration toward melanoma in a xenograft model. Upon activation with IL-2, expression of CXCR3 was up-regulated on human NK cells. Following activation, NK cells had a significantly increased ability to migrate toward the chemokines MIG, IP-10 and I-TAC, as well as toward IP-10 producing melanoma tumor cells in vitro ($P = 0.03$). Infusion of activated NK cells accumulated in IP-10 positive melanoma tumors whereas little or no NK cell infiltration was observed in IP-10 negative tumors ($P = 0.02$). Significantly reduced tumor burden and prolonged survival were observed in IP-10 positive tumor-bearing mice compared to IP-10 negative tumor-bearing mice following infusion of activated NK cells ($P = 0.03$). These results demonstrate specificity in the interaction of IP-10 and CXCR3 expression on NK cells and define IP-10 as a distinct chemokine in accumulation of adoptively infused activated human NK cells in melanoma tumors.

Key Words: Adoptive immunotherapy, Chemokines, NK cells.

Clinical Evidence for the Effectiveness of Traditional Naturopathic Medicines Treatment for Different Types of Cancer in the San Francisco of California United States of America

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Naturopathic medicines are in great demand in both developed and developing countries in primary health care because of their great efficacy and no side effects. Today according to the World Health Organization, as many as 80% of the world's natives depend on naturopathic medicines for their primary health care needs. San Francisco is the largest city in the state of California, and the second-most densely populated large city in the United States of America, and the naturopathic healing systems are still popular here. The studies were conducted during July 2010 to June 2012 using semi-structured questionnaires, open-ended questionnaires, informal interviews, and group discussions with neighborhood naturopathic physicians as well as residents having thorough knowledge about medicinal plants. The data such as local name of

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medicinal plants, plant parts used, application etc. were collected. The voucher samples of the medicinal plants collected during the studies were properly identified with help of floras. The studies were includes information on 36 medicinal plants used for wide range of different types of cancer. Of these 13 medicinal plants are used against malignant tumor, 11 medicinal plants are used against leukemia cancer, 08 medicinal plants are used against breast cancer, 06 medicinal plants are used against colorectal cancer, 05 medicinal plants are used against lung cancer, 03 medicinal plants are used against lymphoma cancer, 03 medicinal plants are used against pancreas cancer, and one medicinal plant is used for blastoma cancer. An assessment of the scientific literatures revealed that preliminary studies conducted on some of the above medicinal plants justify their use to treat specific ailments as practiced by the naturopathic physicians as well as residents. There is enough scope of the amalgamation of these medicinal plants in the main stream of prenatal medicines suggest today after the medicinal plants drug are subjected to the phytochemical and biological screening, together with clinical trials. The studies were to interact with neighborhood naturopathic physicians as well as residents and document their knowledge on medicinal plants, their usage, and the types of cancer treated etc. The formulations mostly contained single medicinal plant instead of multiple medicinal plants. The knowledge evolved for along time through trial and error.

Key Words: Breast cancer, Cancer immunotherapy, Innate immunity.

Myeloid Cell Populations in Patients With Hepatocellular Carcinoma: Correlation With Liver-directed Therapy

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HCC arises in an environment of chronic injury. Tumor macrophages can drive neoangiogenesis, fibrosis and immune suppression, mimicking normal wound healing in a response termed inflammatory resolution. Treatments for HCC may vary in their potential for immune stimulation: resection influences growth factors; ablation causes rapid release of tumor antigen; endoluminal therapy such as intra-arterial TACE and Yttrium-90 produce tumor hypoxia. We quantified monocytes and granulocytes, as well as T-lymphocytes, in peripheral blood of 35 patients with HCC before and 3 weeks after treatment with resection, ablation, or endoluminal therapy, and in patients with liver disease but no HCC (n = 9). Macrophages were quantified by immunostaining with CD68, whereas CD163 was used to identify M2 polarized macrophages in 6 patients who underwent resection. CD8 immunostaining was performed to quantify cytotoxic T cells. Staining was quantified within tumor and nontumor tissue using NIH Image. We found no significant differences in peripheral blood monocyte and granulocyte counts between patients according to size or number of tumors treated, vascular invasion, BCLC or MELD scores, or recurrence rates among the 46 patients studied. There was significantly greater expansion of the granulocyte population following operative procedures for HCC such as resection and laparoscopic ablation than after endoluminal treatments. However, granulocyte expansion was not seen after resection for benign liver masses (n = 3). The small number of patients with more advanced liver disease (Child-Pugh score of B or C) tended to have a greater expansion of monocytes following treatment (ns). There there was not a significant difference in the number of CD163 + staining macrophages or CD8 + cytotoxic T cells within tumor when compared to nontumor. These data provide preliminary evidence of greater pro-inflammatory destructive response after ablation as compared to endoluminal therapy, suggesting that endoluminal therapies may need additional adjuvant stimulation to engender a more active anti-tumor immune response.

Key Words: Tumor milieu, Innate immunity, Macrophages.

Open-label, Randomized Multicenter Phase II Clinical Trial of an Intradermal Toll-like Receptor-2 (TLR2) Agonist Mycobacterium W (CADI-05) Versus Intravesical BCG in Newly Diagnosed Non Muscle Invasive Transitional Cell Carcinoma of Bladder

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Background: TLR2 agonist, mycobacterium w (Cadi-05), a non-specific immunomodulator induces pure Th 1 response, leading to tumor regression following intradermal administration. It is well tolerated and has been found to enhance the efficacy of standard chemotherapeutic agents including but not limited to platinum compounds and taxanes (J. Clin. Onco 29:2011(suppl;abstr 7501)). This study was designed to compare the efficacy and toxicity of Cadi-05 intradermally to intravesical BCG in patients with newly diagnosed non muscle invasive transitional cell carcinoma of the bladder with high probability of recurrence.

Methods: One hundred and twenty treatment naïve patients (pts) with stage T1(69),Ta (47), CIS(4) have been randomized to receive intradermal Cadi-05 (59)or intravesical BCG(61). Cadi-05 was administered intradermally, 0.1 mL on each deltoid following TUR followed by 0.1 mL Cadi-05 every fourteen days for 12 weeks, 0.1 mL Cadi-05 every four weeks for 24 weeks and 0.1 mL Cadi-05 every eight weeks for 24 weeks. The primary endpoint of the study is recurrence rate over two years as evaluated by sonography, cystoscopy and cytology.

Results: Enrollment completed in January 2012 and the median follow up is 63 months. The median age is 57 and 59 years for Cadi-05 and BCG arm respectively. 36 of 69 patients with T1 stage, 21 of 47 patients with Ta stage and 3 of 4 patients with CIS stage received Cadi-05.Of the 120 recruited patients, to date 42 have completed one year follow up without recurrence (21 intradermal Cadi-05 and 21 intravesical BCG).11 of these have completed 100 week follow up (end of study period) and are found to be disease free (6 intradermal cadi-05 and 5 intravesical BCG). Serious adverse events have been seen in two patients receiving intravesical BCG and none in Cadi-05 arm.

Conclusions: These early results (63 wk median follow-up vs. 100 wk planned) suggest that intradermal Cadi-05 may provide a safe therapeutic option to intravesical BCG in the management of non muscle invasive bladder cancer.

Key Words: Genital tumors, Cancer immunotherapy, Innate immunity.

Type I Interferon Signaling in a Mouse *De Novo* Glioma Model

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Glioma accounts for approximately 40% of all primary brain tumors and are responsible for approximately 13,000 cancer-related deaths in the US each year. We have previously demonstrated the protective role of type I interferon (IFN) signaling against glioma progression using a clinically relevant *de novo* mouse glioma model, and identified single nucleotide polymorphisms (SNPs) in human *IFNA* genes associated with the prognosis of patients with glioma (Fujita et al Clin. Cancer Res. 16, 3409-3419, 2010). We have also demonstrated that the A-allele in the rs12553612 SNP, which is associated with better patient survival, allows for IFNA8 transcription via Oct-1 binding, which is absent in patients with the C allele and suggests a molecular mechanism for IFNA8-mediated immune-surveillance against glioma (Kohanbash G. et al Oncoimmunology. 1, 487-492, 2012). However, the molecular factors responsible for inducing the IFN response ("sterile infection") remain elusive. Here we report our initial findings on *in vivo* induction of IFN and its effects on tumor

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microenvironment. To find the cells producing type I IFN, we induced *de novo* glioma by intraventricular transfection of plasmids: pT2/C-Luc//PGK-SB1.a3, *Sleeping Beauty* transposon (*SB*)-flanked pT/CAG-NRas and pT/shp53 in neonatal mice transgenic for IFNβ promoter-driven yellow fluorescence protein (YFP). We detected robust YFP signals in the brain as early as day 30 post induction, when the glioma is still in a dormant or slow growing phase. Our analysis of brain infiltrating leukocytes indicates that CD11b$^+$ myeloid cells are at least partially contributing to the IFN production. Furthermore, our *in vitro* experiments using mouse CD11b$^+$ myeloid cells suggest that glioma-derived genomic DNA and STING (stimulator of IFN genes) in myeloid cells play key roles in the IFN induction. To determine which cells receive the type I IFN signals in the glioma microenvironment, we employed mice transgenic for *loxP-tomato-loxP-EGFP/Mx1-Cre*. When cells in these mice sense type I IFN signals, Mx1-Cre is activated through STAT1, thereby turning from tomato$^+$ to EGFP$^+$ cells. Following the induction of *de novo* gliomas in these mice, we observed approximately 50% of glioma-infiltrating CD11b$^+$ cells as well as CD4$^+$ and CD8$^+$ T-cells turning EGFP$^+$. Further, the level of tomato$^+$ to EGFP$^+$ conversion appeared to be associated with spontaneous regression of gliomas. Our results demonstrate that use of the *SB*-induced *de novo* glioma model in these transgenic mice allow us to determine how type I IFN signals operate in the microenvironment of glioma development. Furthermore, these models will likely contribute to development of IFN-enhancing therapeutic strategies for gliomas.

Key Words: Glioblastoma, Innate immunity, Tumor microenvironment.

Prognostic and Predictive Relevance of Immunological Biomarkers in Colorectal Cancer

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Introduction: The host's immune response may be able to control tumor growth. Down regulation of HLA class I, infiltration of Foxp3+ cells (Tregs) and up regulation of HLA-E and HLA-G are important mechanisms by which immune surveillance can be avoided. The goals of this study were to determine the prognostic and predictive value of these markers in colorectal cancer, define clinical applicable tumor immune subtypes and to define differences between colon - and rectal cancer.

Patients and Methods: The study population consisted of two cohorts. One with 470 Stage I-IV, mainly colon cancer patients all treated for their primary tumor in the LUMC between 1991 and 2001 and one with 532 rectal cancer patients from the Dutch TME trial. TMAs of the colon cancer patients were immunohistochemically stained for HLA Class I tumor expression, for Foxp3+ cells and for HLA-E and HLA-G tumor expression. Tumor immune subtypes were constructed based on underlying biology and quantification of the presence and/or absence of these markers, creating a profile of high, intermediate and low immune susceptible.

Results: In the first colon cohort, loss of HLA class I tumor expression was significantly associated with a better OS and DFS (logrank $P = 0.005$ and $P = 0.014$). The same was true for higher numbers of Foxp3+ infiltrating cells (logrank $P = 0.012$ and 0.039). HLA expression was an independent prognostic factor for survival in multivariate analysis (OS $P = 0.01$, DFS $P = 0.03$). HLA E and HLA G expression were not associated with survival in colon cancer patients. Interestingly, absence of HLA G expression and absence of HLA EG was significantly related to a better OS in patients with loss of HLA class 1 expression with microsatellite stable tumors ($P = 0.022$). High immune susceptible patients were significantly related to a better OS ($P = 0.040$) in patients with microsatellite stable tumors.

In rectal cancer patients the results were different, with presence of HLA G and HLA EG related to a better OS and DFS. (HLA G *P*-value for OS 0.023 and for DFS 0.024, HLA EG *P*-value for OS 0.048 and for DFS 0.040).

Conclusion: The immune system plays an important role in carcinogenesis. Combinations of the immune markers will give more insight in the different immune escape mechanisms and their prognostic role. Tumor immune subtyping can be a promising method for the prediction of prognosis in colorectal cancer. However, preliminary results in our study indicate differences between colon cancer and rectal cancer in interaction with the immune system.

Key Words: Colorectal Cancer, Biomarker, Immune escape.

T Cell-dependent Anti-tumor Activity of NK Cells

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Immune rejection of cancer takes place by the concerted activity of innate and adaptive cell populations. While mechanistic details are available for the innate instruction of the adaptive immune responses, little is known for an equally important adaptive control of the innate immunity. Recent studies in various pathophysiological models of immune rejection, including ours in a mouse cancer model, yield new insights into how an ongoing antigen-specific adaptive immune response can promote an innate response at the site of tissue antigen. We demonstrated in a mouse model of P815 mastocytoma tumors expressing a self "cancer-testis" antigen P1A that effector CD8+ T cells provided a necessary "help" to dormant natural killer (NK) cells in eliciting their antitumor effector function. Live bioluminescence imaging of P815 tumors following adoptive transfer of P1A-specific CD8+ T cells in RAG-/- and RAG-/- gamma chain-/- mice show that NK cell antitumor activity requires cytolytic T cells, whereas T cells can function independent of NK cells. Moreover, the concerted function of T cell and NK cell effector cells operating cooperatively leads to complete tumor regression, including prevention of the development of antigen-deficient tumor escape variants. Thus, promotion of the functional cooperativity of T cell and NK cell effector duets may provide novel therapeutic avenues, with potential to regulate the tumor-associated inflammation that usually overrides the protective antitumor functions of adoptive T cell transfers.

Key Words: Adoptive immunotherapy, Immune escape, Innate immunity.

SINGLE CELL HIGH THROUGHPUT TECHNOLOGIES IMMUNE MONITORING

Use of Immune Monitoring Assays in the Clinical Development of Immunotherapies

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Effective monitoring of anti-cancer immunity is proving vital to the clinical development of immunotherapies. Recently developed assays have the potential not only to measure the effects of therapy on patient immune responses but also to identify patients most likely to benefit from treatment. The prospect of patient selection for personalized medicine is particularly important because immunotherapies typically benefit only a subset of patients, have potentially serious adverse effects and are often costly. We have developed a number of immune monitoring assays to measure humoral and cellular immunity in cancer patients. Our serum profiling assays measure antibodies to proprietary panels of tumor antigens before and after immunotherapy. A patient's trend with respect to serum antibody can be revealing: patients with increasing levels of humoral immunity to key tumor antigens exhibit longer survival times than those whose antibody levels decrease or are

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static following treatment. Crucially, however, baseline antibody signatures have been shown to be predictive of clinical outcome. Our assays for measuring cellular immunity in cancer patients include tetramer staining and counts of myeloid derived suppressor cells (MDSC). We have developed processes for routine measurement of T-cell activation to key panels of tumor antigens to support clinical trials for immunotherapy. In addition the measurement of MDSC is increasingly of interest since it has been shown that abundant levels of this cell type, which is known to suppress the proliferation of T-cells in cancer patients, is predictive for poor clinical outcomes. In this presentation the various methods for monitoring anti-cancer immunity will be reviewed and we will present evidence for their effectiveness in improving clinical development of immunotherapies.

Key Words: Tumor immunity, Tumor associated antigen, Immunotherapy.

CTLA-4 Defines Distinct T Cell Signaling Populations in Healthy Donors and Metastatic Melanoma Patients

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Background: Ipilimumab, an anti-CTLA-4 monoclonal antibody, is approved for treatment of unresectable/metastatic melanoma. CTLA-4 is an immune regulator expressed by regulatory (Tregs) and activated T cells. Ipiliumab treatment benefits only a subset of patients and has been associated with significant side effects and cost. Biomarkers are therefore needed to identify patients most likely to respond. Single cell network profiling (SCNP) is a multiparametric flow cytometry-based assay that quantitatively measures both extracellular phenotypic markers and changes in intracellular signaling proteins in response to ex vivo modulation (Kornblau et al Clin Cancer Res 2010) enabling analysis of cell signaling networks and inter-cellular cross-talk.

Objectives: Examine CD4 + T cell signaling in the context of CTLA-4 expression: a.Between healthy donors and melanoma patient samples b.Between samples from melanoma patients who experienced stable versus progressive disease following ipilimumab administration.

Methods: 10 melanoma pre-treatment samples (4 from pts with stable disease, 5 with progressive disease, and 1 non-assessable) and 3 healthy samples were examined. SCNP analysis of cytokine and TCR signaling nodes focused on the CD4 + T cell subsets defined by intracellular staining of CTLA-4 and Foxp3. Metrics included Equivalent number of Reference Fluorophores (ERF; median fluorescence intensity calibrated per plate), and Uu (the proportion of cells responding relative to basal activity).

Results: Lymphocyte viability was > 90% in healthy donors, but only 7/10 melanoma met the > 60% viability cut-off for analysis. Treg frequencies did not differ between healthy and melanoma samples. Anti-CD3 induced p-CD3-zeta in CD4 + T cells (Table 1). CD4 + T cells from melanoma patients had reduced Uu and ERF compared to CD4 + T cells from healthy subjects. Within CD4 + T cells, melanoma samples signaled highest in the Treg cell subset while signaling magnitude in healthy donor cells was greatest CTLA-4- T cells. Due to low sample numbers, comparison of signaling between responders and non-responders was not feasible.

Conclusions: Signal transduction activities differed between CTLA-4 defined CD4 + subsets, and between healthy and melanoma samples. A planned expansion study will confirm these data, expand upon the biology reported in this study, and assess associations of Ipilimumab response with signaling differences.

Key Words: TCR, Melanoma immunotherapy, CTLA-4.

TABLE 1. Anti-CD3→p-CD3-zeta Signaling in CD4+ T Cell Subsets

Sample	Metric	CTLA-4- Foxp3-	CTLA-4 + Foxp3-	CTLA-4 + Foxp3 + (Treg)
Healthy	ERF	7877 ± 507	6410 ± 937	6142 ± 653
Melanoma	ERF	2011 ± 1332	1855 ± 1587	3604 ± 1885
Healthy	Uu	0.92 ± 0.02	0.82 ± 0.04	0.97 ± 0.01
Melanoma	Uu	0.66 ± 0.08	0.59 ± 0.07	0.78 ± 0.10

Dissection of Anti-CTLA4-induced Cytotoxic T Cell Responses in Melanoma

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There is strong evidence that melanoma-reactive T cell responses induced by immunotherapeutic interventions such as anti-CTLA4 (Ipilimumab) treatment can exert clinically meaningful effects. However, at present we have very little information on how these therapies influence tumor-specific T cell responses. Furthermore, as the number of potential melanoma-associated antigens to which these responses can be directed is very high, classical strategies to map cytotoxic T cell reactivity do not suffice. Knowledge of such reactivities would be useful to design targeted strategies, selectively aiming to induce immune reactivity against these antigens. We have aimed to address this issue by designing MHC class I molecules occupied with UV-sensitive "conditional" peptide ligands, thereby allowing the production of very large collections of pMHC complexes for T cell detection. Secondly, we have developed a "combinatorial coding" strategy that allows the parallel detection of dozens of different T cell populations within a single sample. The combined use of MHC ligand exchange and combinatorial coding allows the high-throughput dissection of disease- and therapy-induced CTL immunity. We have now used this platform to monitor immune reactivity against a panel of 145 melanoma-associated epitopes in patients receiving Ipilimumab treatment.

Comparison of PBMC samples from 26 melanoma patients pre- and post-therapy demonstrates a significant increase in the number of detectable melanoma-associated CD8 T cell responses ($P = 0.006$). Furthermore, kinetic data on T cell responses during Ipilimumab therapy suggest that this broadening generally occurs within weeks after start of therapy. The pattern of reactivities detected is highly patient specific, and this is most pronounced for reactivities directed against cancer/testis antigens. Interestingly, the magnitude of melanoma-specific T cell responses that was already detected prior to start of therapy was not significantly altered ($P = 0.8$). These results establish the pattern of melanoma-specific T-cell reactivity induced by anti-CTLA4 treatment and form a benchmark for evaluation of other immunotherapeutic interventions, like anti-PD1 treatment, that are currently undergoing clinical evaluation. Furthermore, the data suggest that the clinical activity of Ipilimumab may be mostly due to epitope

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spreading, rather than through enhancement of pre-existing immune activity.
Key Words: CD8 + T cells, CTLA-4, Immunotherapy.

Altered Monocyte Phenotype Distribution Predicts Survival in Multiple Myeloma Patients

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Background: Recent advances in the development of prognostic biomarkers for multiple myeloma (MM) have focused on tumor-specific characteristics. Patient-specific characteristics are also important. We have begun to characterize patient peripheral blood immune profiles to identify immune biomarkers. Here we describe our immune profiling approach and provide evidence that certain monocyte subsets can be prognostic for overall survival.
Methods: Sixty-nine patients with newly diagnosed MM had peripheral blood mononuclear cells (PBMC) collected prior to treatment. Thirty-two of these patients had PBMC collected after first-line treatments. PBMC were examined by flow cytometry for twenty cellular immune markers among monocytes and lymphocytes. PBMC from healthy controls (NL, n = 10) were processed and analyzed in the same manner for comparison.
Results: Patient demographics and clinical outcome of this cohort were comparable to those treated at Mayo Clinic over the same time period. Examination of pre-treatment phenotype predictor of survival by univariate analysis identified changes in Treg, CD28 + T-cells, CD4/CD8 ratio, and intermediate monocyte (CD14 + CD16 +) populations. After multivariate analysis adjusting for ISS, age, gender, and treatment groups, intermediate monocytes were the only cell population that remained statistically significant as a predictor of OS (intermediate monocytes ≥ 8%, median OS 4.6 y; < 8%, median OS 8.7 y; $P = 0.02$). HR was 2.26 ($P = 0.01$). Monocyte subset phenotypes were altered in MM compared to healthy controls. The proportions of intermediate and non-classical (CD14-CD16 +) monocytes were increased pre-treatment (intermediate NL 3.3 ± 1.9%, MM 8.5 ± 4.8%, $P < 0.001$; non-classical NL 9.3 ± 3.1%, MM 14.3 ± 7.5%, $P < 0.001$). Post-treatment, only the intermediate monocytes remain elevated while the other subsets were not different from controls. Looking at the magnitude of change in relation to survival we found that found that patients with a low proportion of non-classical monocytes (≤median) that decreased further with treatments had a prolonged PFS (post-treatment 4.86 ± 4.1%, n = 12; median PFS 4.1 y) compared to all others (post-treatment 9.5 ± 5.3%, n = 20; median PFS 2.3 y, $P = 0.04$). HR by multivariate analysis was 0.35 ($P = 0.03$). There was a suggestion of improvement in OS, although this did not reach statistical significance in this limited sample population.
Conclusions: An elevated intermediate monocyte population is an independent pre-treatment predictor of OS in MM patients. Declining proportions of non-classical monocyte during treatment likely improves PFS, and potentially OS. This study supports further study in the role of monocyte subsets in pathobiology of MM and its potential impact on immunotherapy.
Key Words: Tumor immunity, Cellular immunity, Multiple myeloma.

ELISA Tool: An Open-source Relational Database Module for ELISA Experimental Data

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Background: The enzyme-linked immunosorbent assay (ELISA) is widely used in developmental research and clinical immune monitoring due to its flexibility and low cost. The standardized 96-well format and easy-of-execution make it appropriate for high-throughput applications. However, as with other high-throughput techniques, analyzing, auditing, and sharing assay data via spreadsheets or laboratory notebooks can be challenging and error-prone. Additionally, annotating experimental results with relevant clinical information and patient characteristics is tedious and risks confidentiality. We have developed a database tool using Microsoft Access to store and analyze ELISA data in a standardized and detailed format to address these data management issues.
Methods: The ELISA tool was developed within our existing database framework. This open-source tool handles ELISA assay data via linked data tables and SQL queries and allows simultaneous data entry by multiple users. A modular data entry system maintains data on three levels: "Batch" stores assay details (assay date, standard operating protocol, reagents, operator, etc.); "Plate" stores plate name and comments and links the batch data to raw results; and "Results" stores data and annotations (samples, controls, standard curve values) exported directly from the ELISA plate reader. Assay results are captured in both raw and analyzed formats, including dilution series and replicates for each experiment. Key entry fields (antigen, patient, sample ID) are constrained to match entries in related data modules to ensure data integrity. Relational links connect these key entry fields to other data, including sample processing and storage information, de-identified patient demographics, and assay data from other experiments.
Results: The ELISA tool aggregates data from multiple sources within the database. A powerful set of reports and queries organizes and analyzes raw assay data and links experimental data with assay annotations. Results are cross-referenced by key data fields which allow data mining by antigen, patient, and sample ID; links to data from other assays allow sensitivity and specificity calculations for the ELISA assay using matched Western blot data. Standard quality control checks are built into the tool to assess assay performance across multiple experimental variables such as date, operator, or antigen.
Conclusion: Consistently formatted and widely accessible data in a customizable, reliable, and stable database means that our group can use the same information at the same time without concerns over multiple copies of files or incorrect calculations. Annotated raw data can be imported directly, minimizing errors from too much data manipulation, and overall data quality is improved.
Key Words: Cancer immunotherapy, Biomarker.

High-throughput Identification of Biomarkers of Longevity in Ipilimumab-treated Melanoma Patients Using Polychromatic Flow Cytometry

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PBMCs from 12 patients with advanced stage IV melanoma treated with 4 cycles of ipilimumab (Expanded Access Program) were interrogated using a 12-parameter flow cytometry staining panel to delineate discrete memory and effector T cell subsets, and a 10-parameter panel to delineate T regulatory (Treg) cell subsets. Patients received 4 doses of ipilimumab administered at weeks 1, 4, 7, and 10. Analysis was performed on PBMCs collected at week 1 prior to the first dose, at week 7 after two doses, and at week 12. The 12-color memory/effector panel consisted of CD3, CD4, CD8, CCR7, CD45RA, CD27, CD28, ICOS, Ki67, PD-1, NKG2D, and CTLA-4. The 10-color Treg panel consisted of CD3, CD4, CD25, FoxP3, CD45RA, Helios, ICOS, Ki67, NKG2D, and PD-1. Application of Exhaustive Expansion (Siebert et al JTM 2010) generated nearly 20,000 subphenotypes for each parent population (CD4 + and CD8 +) in the memory/effector panel and 6561 subphenotypes in the Treg panel. To identify putative biomarkers of longevity, we analyzed these phenotypes for correlations with survival. As we were particularly interested in predicting patients likely to show a durable response to therapy, we searched for phenotypes for which the baseline readouts or the week 7 fold-change readouts (week 7/baseline) could predict survival > 400 days (7 patients) with 100% sensitivity and at least 60% specificity. We further limited our search to phenotypes that were a credible

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percentage of the overall parent population (≥ 0.5% of CD4 + or CD8 +), and, in the case of week 7 fold-change readouts, showed a fold change ≥ 2 for each of the "long-lived" patients; and to phenotypes in which the readouts for the "long-lived" patients were well separated from the majority of the "short-lived" patients (at least 3 of 5 short-lived patients having readouts ≤ 80% of the minimum readout of the long-lived patients). In the memory/effector panel, we identified 32 phenotypes in which the week 7 fold change matched these criteria. All of these phenotypes were CD4 + and none were CD8 + . The probability of this occurring by random is approximately 1 in 4 billion (0.5^{32}). Additionally, all phenotypes were ICOS + . The probability of this occurring by random is approximately 1 in 2.5 quadrillion (0.33^{32}). Further analysis of the 32 phenotypes suggests that "long-lived" patients rather than "short-lived" patients are more likely to show large fold increases in early activated memory CD4 + T cells. The ability to predict clinical response early in the course of therapy would have important impact on patient care. Thus, we are planning to validate these findings in a larger group of patients.

Key Words: Flow cytometry, Biomarker, Ipilimumab.

Harmonization of Cellular Immunological Biomarkers by an International Network: The Cancer Immunotherapy Immunoguiding Program (CIP)

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Immunomonitoring is an essential aspect in the development of immunotherapies. However, data of cellular immune response assays across laboratories shows high variability and therefore limited comparability The CIMT Immunoguiding Program (CIP), which was founded in 2005 as a working group under the aegis of the Cancer Immunotherapy Association (CIMT), has pioneered the concept of assay harmonization to overcome this hurdle. Currently this network comprises 52 academic and industry participants in Europe and the USA. Through its program of proficiency panels, CIP supports technical validation of in vitro immune assays, and strives to establish high-quality immunological biomarker assessment for guiding the development of new and efficient immunotherapeutics. CIP organizes regular service panels for quality control purposes and exploratory panels for addressing new aspects or techniques. Moreover, CIP has developed and provides cellular reference samples to be used for inter-assay comparibility. In large-scale proficiency panels, CIP has followed a two-step approach to identify and confirm parameters critical for assay sensitivity, specificity, precision and accuracy. These panels also deliver direct feed-back to individual laboratories on assay performance as compared to that of other participants and to internal benchmarks. CIP is sharing its results and data-driven guidelines by peer review publications and on its homepage (http://www.cimt.eu/workgroups/CIP). Results of the most recent proficiency panels will be presented, as well as an update on the activities of the group. While primary effort was focused on the measurement of antigen-specific CD8 + T cells, CIP is extending its area of interest to other immune cells which may play a role in the efficacy of immunotherapy, in particular immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressive cells (MDSCs). Strategies for harmonizing the monitoring of these subsets will be discussed. In conclusion, assay harmonization guidelines obtained by inter-laboratory comparisons (proficiency panels) improve assay performance and reduces assay variability. Moreover, it is easy to implement by laboratories without standardization for not yet validated biomarkers.

Key Words: Cancer immunotherapy, Biomarker, Cellular immunity.

Analysis of Host Immune Cell Infiltration of Immunostained Human Tumors Using a Computer-assisted Pattern Recognition Image Analysis (PRIA) Approach

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Understanding the role of the host immune system in tumorigenesis and how immune modulatory approaches (such as antibodies, vaccines) can be used treat cancer patients is becoming an increasingly important therapeutic area in oncology treatment. However, translation of immunotherapeutic approaches in the clinic will require the development of reliable and robust immune-monitoring strategies for the identification of relevant biomarkers. We have built a platform of immunohistochemistry based assays for the detection of innate and adaptive immune cells in formalin fixed paraffin embedded human tumours, which have been combined with a computer-assisted pattern recognition image analysis (PRIA) approach for the quantification and localization of infiltrating immune cell populations. We studied the intratumoral immune infiltrates in a range of cancers including colorectal and head and neck squamous cell carcinomas (HNSCC). In the HNSCC, histological sections were immunostained for the presence of CD8 + cytotoxic T cells, FoxP3 + regulatory T cells and CD45 + haematopoietic cells. Tumor, stromal and necrotic components were separated in digitally acquired images using GenieTM pattern recognition software, and Aperio image analysis algorithms were applied to quantify infiltrating immune cells in each tissue component. Due to the varied morphology of HNSCC, different GenieTM classifiers were required for each tumor and for each immune marker to accurately segment tumors. Despite this, accurate quantification of inter-individual variation in tumor:stroma:necrosis composition was achieved. Furthermore, immune cell infiltrates predominated in the stroma with a low frequency of immune cells infiltrating tumor cell regions. Analysis of FoxP3:CD8 ratios revealed an immunosuppressive phenotype in the stroma, with elevated FoxP3 + regulatory T cells compared with CD8 + cytotoxic T cells being evident.

These data demonstrate that immune cell infiltrates can be localised and quantified accurately in human tumors using a digital imaging approach enabling assessment of inter- and intra- patient variability in baseline immune cell frequencies and the objective assessment of potential changes upon treatment. The conclusion drawn is that this approach could contribute to biomarker strategies for cancer therapies that modulate the immune system.

Key Words: Biomarker, Tumor infiltration lymphocytes, Tumor microenvironment.

T CELL MANUFACTURE AND POTENCY TESTING

DC Vaccine Potency Correlates With Efficient Induction of Tumor-specific Immune Responses After Vaccination of Advanced Melanoma Patients: Preliminary Data With an in vitro Functional Assay

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DC-based vaccines have been increasingly used in cancer therapy and regulatory agencies, both in USA and Europe, are pressingly requiring the full characterization of the biological activity of cellular therapies (i.e. potency).
Potency assay for DCs should estimate their ability to induce tumor-specific T lymphocytes; however, in vitro stimulation assays are time-consuming and do not actually provide a real measure of their activity of the vaccine in vivo. Allostimulatory capacity in mixed lymphocyte reaction (MLR) have been largely used as a surrogate measure of potency, although it does not separate stimulation due to presentation of alloantigens from that related to actual costimulatory activity of DCs; of note, this latter property has been indicated by the European regulatory agency EMA as a direct measure of DC vaccine potency. In addition, standardized methods able to measure costimulatory activity of DC in a GMP setting have still not fully developed. To test the equipotency of cryopreserved and freshly made DC vaccine, produced in a GMP setting and utilized in patients with advanced melanoma, we developed a modified COSTIM assay, originally reported by Shankar et al.
In this assay, DCs are cocultured for 24 hours with frozen batches of responder allogeneic T cells prestimulated with very low doses of anti-CD3 antibody OKT3; thus, DCs provide to allogeneic responder T cells the costimulatory signals able to trigger their activation. In the original assay, proliferation testing was used as a read-out system; however, proliferation alone does not allow to discriminate DC-induced activation of T cell subsets that can hamper an efficient induction of antitumor immune response by the vaccine (i.e. Tregs).
To avoid this, we utilized IFN-gamma ELISPOT, which can allow to selectively measure a Th1-biased costimulatory ability of DC.
Our data, yet preliminary, showed a positive correlation between vaccine potency and in vivo immunological activity (as assessed by DTH and ELISPOT testings). In addition, freeze/thaw of DC vaccine does not reduce potency up to 4 months of cryopreservation but rather, in 3 of the 4 patients evaluated, potency was even in higher frozen/thawed vaccine than observed in the fresh product.
Key Words: DC-based vaccine, Immunotherapy.

A Novel Human TCR Efficient Cloning System Confers Candidate for TCR Gene Therapy Within 10 days

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Introduction: Antigen (Ag)-specific T-cell therapy or T-cell receptor (TCR) gene therapy is a promising immunotherapy for infectious diseases as well as cancers. High throughput screening system of Ag-specific T-cells and TCR repertoire is requisite for controlling infectious diseases and cancers. Either TCR beta chain or alpha chain repertoire is currently analyzed; however, the availability of a suitable screening system for analyzing both Ag-specific TCR alpha/beta pairs from single T cell is limited. Here, we report an efficient cloning and functional evaluation system of TCR cDNA derived from a single Ag-specific human T cell by which we can obtain TCR cDNAs and determine their antigen specificity within 10 days. We designated this system the hTEC10 system (human TCR efficient cloning within 10 d).
Method: In hTEC10 system, human antigen-specific T cells are detected by staining with antigen-specific MHC tetramers and single cells are obtained by FACS. TCR cDNA is amplified from single cells, cloned into an expression vector, and transduced into the TCR-negative T cell line TG40. The antigen specificity of the TCR is then assessed by staining the transduced TG40 with MHC tetramers and analyzing CD69 expression. This entire process can be performed within 10 days.
Results and Discussion: To evaluate this system, we cloned and analyzed 379 Epstein-Barr virus-specific TCRs from 10 latent healthy donors and showed their CTL activity for antigenic peptide-bearing target cells. In addition, we applied the hTEC10 system to detect and retrieve TCR α/β cDNA pairs from cytokine-secreting CD8 + T cells that were stimulated with a specific peptide. The TCRs obtained from IFN-γ-secreting cells that were stimulated with a specific peptide corresponded with the TCRs recovered from the MHC/peptide tetramer staining of cells from the same donor. Taken together with the CD69 induction assay results, we can use this system with cytokine-secreting CD8 + T cells stimulated with specific peptides without the need to stain with a MHC/peptide multimer. This system may provide a faster and powerful approach for TCR gene therapy for infectious diseases and cancers.
Key Words: TCR, Cancer immunotherapy, EBV.

A HLA-null Cell-based System for the Rapid and Specific Expansion of any CAR + T Cell Independent of Antigen-specific Stimulation

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A novel and promising form of cancer therapy genetically modifies a patient's own T cells in peripheral blood to target and kill their cancer using a chimeric antigen receptor (CAR) directed against a tumor associated antigen (TAA). Growing a therapeutic number of CAR + T cells to treat cancer patients requires an artificial antigen presenting cell (aAPC) to express the TAA which the CAR + T cell targets. Antigen specific growth using a cell based system requires at least a month of intensive monitoring for outgrowth of unwanted cell types. Here we describe a system that decreases the high cost and time associated with manufacturing a different aAPC for each TAA targeted and decreases the time and effort needed to develop, validate, and manufacture large numbers of CAR + T-cells. A HLA null K562 (HnK) was modified to surface express CAR specific to a domain present on all other CARs produced by our lab, which we call G4CAR. When peripheral blood mononuclear cells (PBMC) are genetically modified to express any CAR containing this domain, those CAR + PBMC can be co-cultured with G4CAR + HnK (G4HnK) to specifically grow CAR + T cells independent of TAA. Utilizing G4HnKs to grow CAR + T cells generates a sufficient number of CAR + T cells for therapy in half the amount of time typically needed utilizing antigen-specific aAPC. The T cells consistently grow without competition to a pure population within 2 weeks (> 80% CAR + T cells), and this level of infusion product purity is rarely achieved using current aAPC before one month of T cell co-culture with standard aAPC. Also unique to this system is the capacity to expand antigen non-specific CAR + T cells as a means of antibody independent T cell growth without tissue targeting. This provides a novel method of expanding T cells containing important transgenes (e.g. iCaspase9). In conclusion, we have made a single aAPC cell line capable of expanding any CAR + T cell independent of CAR-specific antigen.
Key Words: T cells, Adoptive immunotherapy, Chimeric receptors.

T CELL MODULATING STRATEGIES

Forcing NF-κB in T Cells Promotes Tumor Rejection

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T cells play an important role in the elimination of tumors. Tumor-specific T cells can be found in cancer patients despite tumor growth. However, in tumor-bearing hosts, tumor-specific T cells can have reduced viability, be intrinsically anergized, extrinsically suppressed, or lack sufficient effector function to successfully reject tumors. Therapeutic strategies aimed at promoting T cell survival and amplifying T cell differentiation/effector function would be extremely desirable as novel cancer therapies.

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NF-κB activity has been reported to be reduced in T cells from tumor-bearing hosts. Our previous results indicate that reduced NF-κB activation results in impaired survival of T cells, decreased Th1 and Th17 differentiation and increased iTreg differentiation. Mice with reduced T cell-NF-κB activity fail to reject cardiac and pancreatic islet allografts in the absence of any pharmacological treatment. We hypothesize that forced activation of NF-κB in T cells should have the opposite effect and promote T cell survival, facilitate Th1/Th17 differentiation and prevent iTreg differentiation, which would be beneficial to reject tumors.
We generated mice expressing a constitutively active form of IKKβ (CA-IKKβ) in T cells. Ectopic expression of CA-IKKβ resulted in phosphorylation of NF-κB. Transgene expression was limited to CD4 + , CD8 + and NKT cells and T cells showed increased NF-κB activation and nuclear translocation. T cell numbers were comparable to littermate controls, but CA-IKKβ mice had fewer Tregs and increased frequency of activated T cells that produced IFNγ upon re-stimulation. When B16-SIY melanoma cells were injected subcutaneously, tumors grew progressively in control littermates, whereas they were rejected by mice expressing CA-IKKβ in T cells. CA-IKKβ expressing T cells were necessary for tumor control, as shown by antibody-mediated depletion of CD4 + and CD8 + T cells. Furthermore, adoptive transfer of CA-IKKβ-expressing, but not wild-type, T cells into immune-compromised (RAG-deficient) hosts prior to inoculation of tumor cells was sufficient for tumor control. Tumor control was associated with a massive increase in the number of tumor-specific IFNγ-producing CD8 + T cells and IKKβ-CA + CD8 + T cells were able to control tumor growth in the absence of CD4 + T cell help.
Interestingly, on the other hand, IKKβ-CA + CD4 + T cell help was sufficient to induce tumor control by WT CD8 + T cells. Finally, enhanced tumor control was observed in immune-competent mice when fewer than 5% of T cells expressed CA-IKKβ.
Our results demonstrate NF-κB to be at the cross-roads of major T cell fate decisions that uniquely synergize for control of tumor growth and may be translatable to the clinic.
Key Words: T cells, Melanoma.

Induction of CXCL9/MIG Expression in the Tumor Microenvironment Promotes Protective Anti-tumor Immune Responses

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Despite intense efforts to design strategies inducing protective immune responses to tumors, the success of cancer immunotherapy remains extremely limited. Major factors limiting immune responses to tumors include insufficient activation of tumor-reactive effector T cells, poor intra-tumor recruitment of effector T cells, and/or suppression of their functions within the tumor microenvironment. One mediator involved in effector T cell priming and recruitment to inflammatory sites is the T cell chemoattractant chemokine CXCL9/Mig. Our recent studies in murine models of aggressive skin cancer (cutaneous fibrosarcoma and melanoma) have indicated that these tumors cease the production of CXCL9/Mig and become more resistant to T cell-mediated immunity than Mig-expressing tumors. Here we tested if the restoration of Mig expression in the tumor microenvironment promotes protective anti-tumor immune responses.
Constitutive expression of Mig in tumor cells induced by the delivery of Mig-encoding viral vector converted poorly immunogenic Mig-deficient tumor cells into highly immunogenic variants and induced potent tumor-specific CD4 and CD8 T cell responses capable of complete tumor elimination and subsequent protection to tumor re-challenge. Furthermore, the delivery of Mig-overexpressing tumor cells into mice with established cutaneous tumors induced systemic anti-tumor immune responses that effectively suppressed growth of these tumors. This robust therapeutic effect correlated with the presence of dendritic cells (DC) in the tumor-draining lymph nodes that were very potent in the activation of tumor antigen-specific T cells. These results suggest an important role for CXCL9/Mig in the activation of tumor-reactive effector T cells, at least partially, through changes in numbers and/or functions of tumor antigen-presenting DC populations. We are currently testing the mechanisms underlying the role of CXCL9/Mig in activating and optimizing tumor-specific T cell responses that eliminate cutaneous tumors and protect from tumor recurrence.
Key Words: T cells, Cancer immunotherapy, Chemokines.

Rapamycin Treatment Endows Car-engineered CD8 + Effector T Cells With Memory-like Properties Resulting in Enhanced in vivo Engraftment

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Adoptive transfer of tumor-specific T cells is proving to be an effective method for treating established tumors. However, naturally occurring tumor-specific T cells are rare in cancer patients and require extensive ex vivo manipulation to generate a sufficient bolus of cells for treatment. To facilitate the production of tumor-specific T cells, we have engineered bulk T cells to express chimeric antigen receptors (CARs) that are specific for tumor antigens. CARs confer tumor specificity via an extracellular antigen recognition domain, often a single-chain antibody, and trigger T cell effector function via intracellular signaling domains derived from the T cell activating proteins CD3zeta and CD28. Several reports have suggested that T cells with a memory phenotype may be preferential for adoptive transfer because they demonstrate enhanced engraftment, proliferative capacity and subsequent anti-tumor activity in vivo when compared to T cells with an effector phenotype. Signaling via mTOR has been shown to suppress memory development. Therefore, to generate CAR-engineered T cells with a memory phenotype, we included the mTOR inhibitor, rapamycin, during retroviral transduction of murine splenocytes with a CAR. The resultant population manifested a unique phenotype. The cells expressed characteristics of memory cells (elevated CD62L, eomes expression, and suppressed cytokine production) while also displaying hallmarks of effector cells (elevated granzyme B and t-bet expression). Rapamycin treatment of CAR-engineered CD8 + T cells prior to adoptive transfer resulted in enhanced engraftment in tumor-bearing animals in comparison to cells cultured without rapamycin. In vivo impacts of rapamycin treated CAR T cells on tumor growth are currently being evaluated. These experiments investigate a novel method for culturing CAR-engineered T cells, with the goal of enhancing in vivo engraftment and functionality, and thus provide further insight into adoptive transfer as a cancer immunotherapy.
Key Words: T cells, Adoptive immunotherapy, Chimeric receptors.

Optimizing the Therapeutic Potential of PD-L1 Blockade as a Single Agent and Through Combination Therapy

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PD-L1, through engagement of the inhibitory receptor PD-1, impairs the capacity of chronically activated T cells to proliferate, produce cytokines, or effectively kill target cells in response to their cognate antigen. Expression of PD-L1 is prevalent among human tumors and can impede anti-tumor immunity resulting in immune evasion by tumor cells. Recent clinical data demonstrate the ability of antibody blockade of the PD-1/PD-L1 pathway to induce tumor regression in multiple tumor types. Here we describe the activity of a monoclonal antibody that targets PD-L1 and inhibits its interaction with both known receptors, PD-1 and B7.1. The IgG1 antibody was engineered with an Fc modification that abolishes FcγR binding in order to reduce antibody-mediated killing of Ag-experienced T cells that express elevated levels of PD-L1 or

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susceptible tissues that harbor higher levels of PD-L1 expression. In vitro, the Fc-modification in IgG1 prevents antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells at concentrations of 20 μg/mL; in contrast, an IgG4 isotype antibody, often considered to be ineffective in mediating efficient ADCC provides significant killing at concentrations as low as 0.2–2 μg/mL. Targeting PD-L1 with an Fc-effectorless antibody is expected to optimize both efficacy by protecting PD-L1hi tumor-infiltrating lymphocytes from depletion and safety by reducing tissue damage and potential presentation of auto-antigens from PD-L1-expressing tissues targeted by the antibody. In syngeneic tumor studies conducted with a human/mouse chimeric Fc-modified antibody, anti-tumor activity occurs rapidly and can translate into durable tumor-specific immunity. Significant synergy is observed combining PD-L1 blockade with selected chemotherapeutics, small molecule inhibitors of oncogenic pathways or blockade of VEGF. Some treatments that enhance anti-tumor efficacy of anti-PD-L1 can impede responses of alternative T cell-enhancing therapies, highlighting the need to understand mechanism for predicting effective combination regimens. Finally, immune-enhancing activity of BRAF V600 mutation inhibitors provides support for combining anti-PD-L1 with vemurafenib in patients with BRAF V600-mutant melanoma. The broad development potential of the engineered IgG1 anti-PD-L1 antibody, both as a single agent and in combination with tumor-targeted therapies will be discussed.

Key Words: Cancer immunotherapy, PD-1.

Memory CD8 + T Cells Induce Precocious Effector Differentiation of NAïVE CD8 + T Cells in a FASL-FAS Dependent Manner: A New Mode of T-T Lymphocyte Interaction and Cross Talk

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Naïve (T_N), stem cell memory (T_{SCM}) and central memory (T_{CM}) CD8 + T cell subsets have been shown to confer superior engraftment and anti-tumor efficacy relative to more differentiated effector memory (T_{EM}) and effector (T_{EFF}) CD8 + T cells. However, whether the presence of more differentiated T_{EM} and T_{EFF} negatively influence the potential of their less differentiated counterparts remains unknown. Herein, we describe a previously unrecognized interaction between T_N and memory CD8 + T cells (T_{MEM}) that directly enhanced the effector differentiation of T_N via non-apoptotic Fas signaling resulting in downstream activation of the pro-differentiation Akt pathway. Using congenic markers to indelibly fate-track CD8 + T cell subsets, we found that the presence of T_{MEM} during priming caused T_N to differentiate more rapidly than T_N activated alone, a process we have termed precocious differentiation. In addition to an accelerated loss of the lymphoid homing markers CD62L and CCR7 and the naïve-associated transcription factors (TFs) Tcf7, Lef1, and Klf2, T_N primed with T_{MEM} acquired higher levels of IFNγ, granzyme B, and the effector-associated TFs T-bet and Blimp-1. Investigations using microarray analysis of re-isolated T_N and T_{MEM} demonstrated the pervasiveness of this phenomenon as T_N transcriptionally associated with T_{MEM} by hierarchical clustering within 18 hours of activation. This process was TCR-ligation dependent, cell-dose dependent, and required cell-cell contact as it was entirely abrogated by physical separation of T_{MEM} from T_N using a semi-permeable membrane. Mechanistically, disruption of FasL-Fas signaling either by antibody blockade of FasL or use of T_N deficient in the Fas receptor prevented precocious differentiation while provision of exogenous FasL trimer in the absence of T_{MEM} recapitulated this phenomenon. To interrogate the biologic significance of precocious differentiation, we adoptively transferred T_N activated alone or in the presence of T_{MEM} to treat hosts bearing B16 melanoma tumors. Naïve cells primed either *in vitro* or *in vivo* with T_{MEM} acquired a terminally differentiated phenotype upon transfer, as evidenced by low levels of CD27 and high KLRG1 expression, and exhibited significantly impaired persistence and antitumor activity compared with T_N primed alone. These findings provide evidence that more differentiated T_{EM} and T_{EFF} actively corrupt the full therapeutic potential of less differentiated anti-tumor T cells and demonstrate that their physical separation is required for optimal efficacy of adoptive T cell-based immunotherapies.

Key Words: Melanoma immunotherapy, Adoptive immunotherapy, Memory CD8 + T cells.

Identification of Immunogenic Epitopes From Cancer Stem Cell Antigens for the Design of Multi-epitope TH1 CD4 + T Cell Vaccines Against Breast Cancer

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Background: Cancer stem cells may drive the initiation and maintenance of malignancy and metastasis. They express epithelial-to-mesenchymal transition (EMT) proteins that are potential targets for immunotherapy. Previous work from our group has demonstrated that vaccination with immunogenic epitopes of tumor-associated antigens such as HER2/neu can generate persistent antigen-specific immunity which may in turn provide durable protection against relapse. Proteins associated with breast cancer stem cells (bCSC) and EMT may be ideal candidates for breast cancer vaccines if they are properly immunogenic. We have devised an efficient method for identifying putative promiscuous high affinity binding MHC class II epitopes from bCSC/EMT expected to be immunogenic across a wide range of individuals (i.e. universal epitopes).

Methods: We conducted a systematic literature review to select bCSC antigens with the following characteristics: (1) over-expression in breast cancer; (2) association with EMT; (3) association with breast cancer cells; and (4) independent poor prognostic indicators via univariate or multivariate analysis. Fifteen candidate antigens (including cell surface molecules, transcription factors, and intracellular signaling molecules) were analyzed using an in silico epitope prediction algorithm to identify high-affinity epitopes. Epitopes were synthesized as 12-26mer peptides and tested for immunogenicity in vitro using the ELISPOT assay to measure antigen-specific cytokine (IFNγ and IL-10) secreting cells in PBMC from 20 breast cancer donors and 20 healthy controls.

Results: We present here the results from 49 peptides identified from the first 6 bCSC/EMT antigens. The majority (45/49) of the peptides elicited IFNγ ELISPOT responses in either healthy or cancer donor PBMCs, while 3/49 elicited only IL-10 ELISPOT responses and 1/49 elicited no detectable responses. The majority (32/49) elicited both IFNγ and IL-10 responses. All peptides were evaluated for IFNγ/IL-10 cytokine profiles across all donors. Epitopes which generated the broadest-based (highest incidence) and most potent (greatest magnitude) IFNγ responses with low or no immunosuppressive IL-10 responses remain candidates for inclusion in the final vaccine product.

Conclusions: Our results demonstrate the feasibility of selecting immunogenic peptides (via in silico and in vitro methods) that preferentially elicit antigen-specific IFNγ responses in both normal and cancer PBMC. These responses may be protective when generated in a vaccine setting given the initial criteria for antigen selection. We will test the selected universal epitopes of bCSC/EMT antigens in mouse models to further validate this hypothesis.

Key Words: Cancer vaccine, Tumor associated antigen, Immunotherapy.

Blockade of PD-L1 Mediated Immunosupression for Cancer Therapy - MEDI4736, Monoclonal Antibody Discovery and Preclinical Development

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PD-L1 (B7-H1) is part of a complex system of signalling checkpoints that are involved in controlling T cell activation and helps to regulate normal immune responses, through its interaction with the PD-1 and CD80 receptors. A high proportion of tumor infiltrating T cells over express PD-1, as a result of chronic antigenic stimulation. Many tumors take advantage of this by up-regulating PD-L1, allowing them to hijack the PD-1/PD-L1 signalling axis to inhibit the anti-tumor T cell response and evade detection and elimination by the host immune system. Anti-PD-L1 antibodies, which block the interaction of PD-L1 with its receptors, have the potential to overcome this inhibitory signalling and re-instate anti-tumor immunity.

Using hybridoma technology and high throughput screening MedImmune has identified a series of fully human antibodies specific for human PD-L1. Further characterisation of these antibodies led to the identification of a single high affinity antibody, MEDI4736, with the ability to relieve PD-L1 mediated suppression of T-cell activation in vitro and to enhance sub-optimal T-cell activation in a mixed lymphocyte reaction. In vitro testing shows that MEDI4736 does not trigger non-specific cytokine release in whole blood, and is only able to activate T cells in the context of an active T-cell receptor signal. A surrogate anti-mouse PD-L1 antibody shows significant anti-tumour activity in a syngeneic model when dosed in combination with chemotherapy. Similarly MEDI4736 is able to inhibit tumour growth in a novel in vivo xenograft model, via a mechanism that is dependent on the presence of tumour specific human T cells. These results demonstrate MEDI4736 is a selective antagonist of PD-L1 and may be a promising approach to targeting immune escape mechanisms observed in tumours.

Key Words: PD-1.

Inhibition of Glycolytic Flux Enhances CD8 + T Cell Memory, Stemness and Anti-tumor Function

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The ability of tumor-reactive T cells to eradicate tumors following adoptive transfer correlates with their capacity to robustly proliferate and persist for a long period of time. These qualities are predominantly found in naïve and less differentiated memory cells such as memory stem cells (TSCM) and central memory cells (TCM) but the determinants of these attributes are poorly understood. While numerous transcriptional and epigenetic changes have been implicated in the generation and maintenance of various T cell subsets, it remains unclear whether changes in cellular metabolism have an influence on T cell fate and function. We found that naïve T cells, which rely on fatty acid oxidation as a primary source for ATP generation, dramatically shifted to a glucose metabolism following antigen stimulation and effector differentiation. Sorting effector cells based on their glucose uptake revealed that cells incorporating less glucose had an enhanced ability to engraft and establish long-term memory following adoptive transfer suggesting that glucose metabolism might determine T cell fate decisions. Specific blockade of glycolysis during T cell priming by the hexokinase inhibitor, 2-deoxyglucose (2-DG) prevented effector differentiation resulting in the generation of memory CD8 + T cells. Furthermore, we found that genes that transduce Wnt β-catenin signaling that are related to T cell stemness such as T cell factor 7 (Tcf7) and lymphoid enhancer binding-factor 1 (Lef1) were dramatically increased in CD8 + T cells sorted for low glucose and also in 2DG treated CD8 + T cells compared to untreated controls. Most importantly, we observed a 100-fold increase in the frequency of secondary memory CD8 + T cells detected in lymphoid and non-lymphoid organs and an enrichment of TCM over senescent KLRG1 + T cells upon adoptive transfer of 2DG-treated cells compared to controls. In tumor-bearing mice, 2-DG treated cells exhibited increased tumor-infiltration, cytokine functionality, and resulted in the regression of large-vascularized tumors. 2-DG treatment led to sustained activation of Foxo1, a transcription factor that promotes T cell memory, through inhibition of the mTOR pathway. These findings identify glycolysis as a key metabolic pathway that limits T cells from entering into the memory pool and provide a basis for the rational design of new adoptive immunotherapies through the specific modulation of glucose metabolism.

Key Words: Adoptive immunotherapy, Memory CD8 + T cells, Immune-mediated tumor rejection.

TARGETED THERAPIES AND ANTI-TUMOR IMMUNITY

Results of Using Immunomodulators in Combined Treatment of Low Rectal Cancer With the Liver Metastasis

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Background: Improvement of results combined treatment of metastatic low rectal cancer by using chemotherapy with target therapy, immunotherapy and ozonotherapy.

Materials and Methods: We have taken randomized study on 45 patients in period during 2006–2012 year from them in 1st group (23 patients) included patients that have taken Bevacizumab(5 mg/kg intravenous infusion every 7 d), immunomodulator by intramuscular injection of Transfer Albuminatus Factor (TAF) and intravenous infusion of ozonized liquor with chemotherapy(CT) by scheme FOLFOX -4(Oxaliplatin-100 mg/m^2 on 1st day, Leukovorin-200 mg/m^2 on 1st day, 5Fluorouracil(5FU)-400 mg/m^2 intravenous intermittent administration on 1st day, than 5FU - 2,4 - 3,0 g/m^2 48 h flat continuous infusion) with interval 3 weeks. 2nd group (22 patients) taking CT by similar scheme of intravenous infusion without immunotherapy and target therapy, with interval 3 weeks.

Results: All patients analyzed for toxicity. Main G 2-3 side - effect were: neutropeniya on 1 group- 4,3%, II group-45,5%, diarrhea-4,3; 18,2%, stomatitis-4,3%; 9,1%, neurotoxity-0; 4,5%, deep venous thrombosis-0%; 4,5%, hypertension - 4,3%; 13,6%, cardiac ischemia - 0; 4,5%. No toxic deaths have occurred. All patients have been evaluated for response and we observed 7 cases in I group (1 in II group) complete and 13(15 in II) partial response rate and 3 stable disease(4 in II group). Up to now 18 patients in I group and 6 in II underwent to post-CT surgical resection of metastases with curative intent and 14; 2- R0 resection have been performed. At median follow-up of 16,3 and 12,3 months, 9 and 18 patients have progressed and median progression-free survival (PFS) is 13,1; 10,1 months with an actual PFS at 10 months of 72%; 46%. To date 7; 16 patients have been died and median overall-survival (OS) has not yet been reached.

Conclusion: Using endoarterial chemotherapy can be safely combined with ozonotherapy, immunotherapy and target therapy without increasing toxicities no causing unforeseen adverse events. Preliminary data in term of partial response rate, secondary resection of metastases and PFS are promising increase effect of CT.

Key Words: Colorectal cancer, Chemotherapy, Combination immunotherapy.

EN2: A Candidate Antigen for the Development of Targeted Therapies in Breast Cancer

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Engrailed (En) is a homeodomain-containing transcription factor with a multifunctional role in neural development. In vertebrates there are two engrailed genes; En-1 and En-2, each with their own specific functions. A unique feature of these proteins is their ability to regulate both transcription and translation at different stages of normal development. EN2 is normally involved in brain development in the embryo then silenced in adulthood. However, initial studies have shown that EN2 is over-expressed in a variety of cancers including prostate, ovarian and colon carcinomas. Furthermore, EN2 has been characterised as an oncogene in breast cancer due to its aberrant expression and its tumor-promoting role in human breast cancer. The oncogenic nature of EN2 and its over-expression specifically in tumours makes it an ideal immunotherapeutic target. To date we have confirmed by immunohistochemistry on high density tissue arrays that EN2 protein is expressed in > 90% of early and late-stage breast cancer including triple negative breast cancers. In addition, we have shown that EN2 protein is immunogenic. Cell-mediated immune responses to EN2 have been generated in vitro from HLA-A2 positive healthy donors and the EN2 epitopes inducing the response identified. Importantly, these EN2 specific T cells were able to recognise and kill breast cancer cell lines in an HLA-restricted manner. Preliminary work has also demonstrated an antibody response to EN2 in a significant proportion of breast cancer patients derived from three independent cohorts. To our knowledge this is the first study demonstrating the human immune reactivity to this protein EN2. The key role of EN2 in breast cancer development, its over-expression specifically in breast tumours and its immunogenicity makes it an interesting antigen to exploit as a novel target for breast cancer immunotherapy.

Key Words: Cancer vaccine.

Trends in Circulating Tumor Cells (CTCS) in Multiple Adjuvant (ADJ) Trials of HER2-Specific Peptide Vaccines (PV) in Breast Cancer (BRCA) PTS

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Background: CTCs are an independent prognostic factor of overall survival in metastatic BrCa and data suggests a role for CTCs predicting recurrence in pts with non-metastatic BrCa. We are conducting phase II trials evaluating 3 HER2-specific vaccines (E75, AE37, GP2) in the adj setting and have previously published "proof of principle" data suggesting a potential role for CTCs as a marker of response to adj immunotherapy. This study was undertaken to evaluate updated data on CTCs in these trials.

Methods: Node + or high-risk node-, disease-free BrCa pts with any level of HER2 expression were enrolled after standard treatments. In the AE37 & GP2 trials, pts were randomized to either peptide + GMCSF(vaccine group,VG) or GMCSF alone(control group,CG). In the E75 trial, HLA-A2/A3 + pts were assigned to the VG and HLA-A2/A3- pts were followed prospectively as a CG. VG pts in all trials received six, monthly intradermal inoculations in the primary vaccine series(PVS) followed by booster inoculations every 6 months(B1-B8). CTCs were enumerated from blood samples using the CellSearch(Veridex). After establishing baseline CTCs, those with ≥ 1 CTC had subsequent measurements taken at R3, R6 and with each booster. Pts with multiple data points were divided into those with increased/stable(I/S) or decreased(D) CTCs. Immunologic response in the E75 trial was measured in vivo with delayed-type hypersensitivity (DTH) and in vitro with dimer assays.

Results: Combining all trials, CTCs were measured in 96 pts(74VG, 22CG) with 56 (57%) having ≥ 1 baseline CTC. 44(39VG, 5CG)/56 had > 1 CTC data point. VG pts were more likely to have a decrease in CTCs than were CG pts(59% v 20%, $P = 0.16$). Analyzing E75 pts alone, 26 had > 1 data point. 16/26 pts had decreased CTCs with an average decrease of 3.06 ± 0.93(SEM). The average number of CTCs decreased from R0 to R6 and all post-PVS time points, but rarely to 0(R0:4 v R6:0.51; preB1:1.6;postB1:0.4, $P = 0.005$;preB2:0.72, $P = 0.03$;postB2:0.42; preB3:1.37;postB3:0.75; preB4:0.57;postB4:0.67; preB5:1;postB5:0.50;preB6:0.50;postB6:1.33;preB7:1;postB7:0.75;preB8:1;postB8:1). Compared to I/S pts, D pts had increased post-PVS DTH and dimer response(DTH I/S:12.5vD:24, $P = 0.03$;dimer I/S:0.21vD:0.81, $P = 0.07$).

Conclusions: Adj BrCa vaccines may decrease the number of CTCs and our data suggests a correlation with standard immunologic response assays. CTCs rarely disappeared, lending credence to the theory that BrCa is a chronic disease. Monitoring CTC trends may be clinically useful in the adj setting as a surrogate for response to PVs.

Key Words: Breast cancer, Cancer vaccine.

IMCGP100: A Novel BI-SPECIFIC Biologic Immunotherapy for the Treatment of Malignant Melanoma

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In recent years significant advances in the treatment of metastatic melanoma have emerged. Small molecule drugs provide potent short-term responses for a significant proportion of the patient population; for a minority of patients, immunotherapy has elicited long-term responses with the promise of a cure. Despite these advances, long-term remission for the majority of patients remains elusive and much effort is focussed on combination therapies attempting to bring together the potency of small molecule drugs with the durability of immunotherapy.

IMCgp100 is a novel bi-specific immunotherapy comprising a soluble, affinity- enhanced, T cell receptor (TCR) specific for the melanoma-associated antigen gp100, fused to an anti-CD3 specific antibody fragment (scfv). The engineered TCR portion of the drug targets the gp100 peptide 280-288 antigen, which is over- expressed and presented by HLA-A2 on the surface of melanoma cells, thereby effectively coating these cells with CD3-specific antibody fragments. The anti-CD3 scfv portion captures and redirects any T cells in physical contact with the melanoma cell to kill it. Within the T cells immune repertoire, T effector memory cells and Temra T cell subpopulations are the most potent in eliminating the melanoma cells while T central memory and naïve cells are induced to proliferate and to become effector cells capable of potent killing. In vitro, IMCgp100 potently redirects T cells from the blood of late stage cancer patients to target melanoma cells exhibiting substantial HLA-down regulation, even in the presence of high numbers of regulatory T cells. The killing of multiple targets by a single T cell is observed within hours, and is associated with the release of pro-inflammatory cytokines. Apoptotic melanoma cells are promptly phagocytosed by dendritic cells that cross-present gp100 and other melanoma antigens to the immune repertoire. Thus, IMCgp100 demonstrates the potential to elicit potent short-term responses and trigger longer-term anti melanoma durability in vivo. IMCgp100 is currently under investigation as part of a Phase 1 dose-finding study in patients with unresectable Stage III/Stage IV malignant melanoma. We have also performed a Phase 0 trial in which IMCgp100 is injected directly into tumours to assess pharmacodynamic activity in human lesions. The Phase 1 study is actively enrolling and preliminary clinical data will be presented.

Key Words: Melanoma immunotherapy, Targeted therapeutics.

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Immune Suppressive Activity Mediated by Oncogenes and Loss of Tumor Suppressor Gene Activity

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Modulation of MHC class I surface antigens represents a major mechanism of murine and human tumor cells to escape T cell-mediated immune responses, which is associated with deficiencies in molecules of the MHC class I antigen processing and presentation machinery (APM). The underlying molecular mechanisms of these abnormalities are mainly due to deregulations at different levels rather than structural alterations of APM components. Using HER-2/neu-transformed cells, a reduced MHC class I surface expression was demonstrated, which was mainly mediated by a transcriptional downregulation of the expression of various APM components, such as peptide transporter subunits, the low molecular weight subunits and tapasin. Site-directed mutagenesis of transcription factor binding sites in the APM promoters showed for first time that E2F1, p300 and CREB play a key role in the transcriptional repression of TAP, tapasin and LMP subunits, which was associated with a reduced transcription initiation. The link between APM component, E2F1 as well as CREB expression was confirmed by (i) shRNA-mediated inhibition of E2F1 and CREB in E2F1- and CREB-overexpressing cells and (ii) overexpression of E2F1 and CREB in non-malignant cells. CREB silencing in HER-2/neu-transformed cells was associated with an enhanced MHC class I and APM component expression as well as a reduced tumor formation. There exists recent evidence that not only oncogenes, but also tumor suppressor genes are able to modulate MHC class I surface expression. Biglycan (Bgn), a member of the small leucine rich proteoglycan family, has been shown to be downregulated upon oncogenic transformation. This was directly linked to MHC class I deficiencies, while reconstitution of Bgn leads to an induction of components of the MHC class I pathway and loss of tumorigenicity. In addition, blocking of the HER-2/neu-controlled down-stream signaling pathways by distinct inhibitors was also able to restore the immunogenicity of HER-2/neu-overexpressing tumor cells suggesting that the modulation of tumor suppressor and oncogene activity as well as their signal transduction pathways might lead to the development or design of novel therapies.

Key Words: Immunosuppression, Cancer immunotherapy, Immune escape.

Generation of an Antitumor Response and Immunity Using a Small Molecule Drug (PV-10)

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PV-10 (10% Rose Bengal in 0.9% w/v NaCl solution) recently has been used to chemoablate a wide variety of tumors in human clinical trials and in animal patients when delivered by intratumoral injection. PV-10 exhibits antitumoral activity targeted only on diseased tissue while sparring normal tissue. Additionally, PV-10 stimulated the removal of remote untreated tumors by immune-mediated antitumor responses including brain and lung metastases.. Therefore, we investigated the mechanism by which this remote "bystander " effect was produced using immunocompetent and incompetent mice. Tumor models examined include: hepatocellular carcinoma, melanoma, pancreatic and colon adenocarcinomas. PV-10 was found to chemoablate all tumors tested with no apparent side effects. No tumors resolved in control mice injected with diluent only. Durable immunity was produced to the same tumor that was ablated. However, tumors of different origin could be established in treated mice. Immunity to establishment of a tumor could be transferred using spleen cells from mice whose tumors had been previously chemoablated. Remote untreated tumors in the opposite flank of mice were removed or reduced in size in imunocompetent mice (e.g. HCC 8/9). No remote tumors have ever been observed to resolve in nude mice when a second tumor was treated by PV-10 chemoablation. In conclusion, remote removal of untreated tumors is dependent on an immune mechanism requiring T-cells. We hypothesize that production of an immunotherapy/vaccine like response using a small molecule drug is thought to require: 1) intratumoral route of injection that generates rapid massive tumor killing, 2) rapid clearance of drug from normal tissue, 3) antitumor effects targeted only to tumor tissue, 4) reduction of tumors via production of autophagy/apoptosis.

Key Words: Tumor immunity, Cancer vaccine, Apoptosis.

Pralatrexate (Folotyn TM) May Exert an Anti-marginal Zone Lymphoma (MZL) Effect Through an Immune Mediated Mechanism

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MZL are subtypes of indolent B-cell non-Hodgkin's Lymphoma (NHL) some of which are characterized by T cell-dependent B-cell activation and deficient cytotoxic control of B-cell growth. Pralatrexate (P) is a folate analogue metabolic inhibitor indicated for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma (PTCL). While P has notable single agent activity in PTCL, its single agent activity in B-cell NHL subtypes has been modest per early clinical reports however systematic clinical trials are ongoing to formally characterize its role in such disease entities. We present a unique case of MZL that shed a light on a possible Pralatrexate related immune mediated anti-MZL effect. Our patient is 53 year old male who was diagnosed with relapsed MZL after he presented with generalized lymphadenopathy (LN) and subcutaneous biopsy proven MZL nodules. The patient was enrolled on one of the Pralatrexate trials in B-cell lymphoma where he received P at 30 mg/m^2 IVP weekly for 3 consecutive weeks every 28 days. Initially the patient's subcutaneous nodules (SN) responded with a notable decrease in size and numbers. CT scans obtained after 2 cycles revealed stable disease per IWG criteria. He went on to receive his 3rd cycle per protocol but presented soon after with what seemed to be rapid progression of his generalized SN and LN with a notable increase in size and numbers on physical exam and CT/PET imaging. The patient was deemed to be a treatment failure and P was discontinued. A biopsy of his largest axillary lymph node revealed significant reactive component with residual MZL. At that time patient declined immediate therapy opting to defer it till symptomatic progression. Surprisingly, his SN and LN stabilized and almost completely resolved by the 4th and 7th month respectively. Moreover, he remains to be in CRu a year after he was deemed to have progressed though he received no further therapy. The appearance of pseudo-progression prior to his durable CRu point to an immune mediated mechanism of action. Given the significant anti-T cell activity of P, we propose that P may act through an immune mediated pathway by eliminating T cell-dependent B-cell activation and restoring anti-MZL cytotoxic activity.

Key Words: Tumor immunity, B cell, Lymphoma.

A Novel Assay to Measure the Immunogenicity of Different Ionizing Radiation (IR) Regimens

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Purpose: Recent evidence suggests that IR induces immune-mediated systemic effects (Formenti, Lancet Oncology 2009) that were described as abscopal (Demaria, IJROBP 2004).

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Immunogenic cell death (ICD), characterized from standard therapy by Kroemer and Zitvogel, is orchestrated through three indispensible pathways: 1) surface translocation of calreticulin (CRT, an ER resident protein) and the extracellular release of 2) high-mobility group protein B1 (HMGB1, a non-histone nuclear protein) and 3) ATP.

We developed an assay to rapidly detect IRs contribution to ICD with engineered cell lines from parental TSA cells (BALB/c murine mammary cancer).

Methods: CRT fusion protein was detected by fluorescent microscopy in TSA cells transfected with the pEZ-M02 vector containing the CRT-HaloTag-KDEL construct (Fig. 7A). ER and membranous localization of CRT fusion protein was validated with incubation of membrane permeable R110Direct ligand or membrane impermeable alexa fluor 488 ligand, respectively.

HMGB1-GFP and HMGB1-RFP nuclear localization was detected by fluorescent microscopy in TSA cells transfected with the pCMV6-AC-GFP or pCMV6-AN-RFP vectors with an HMGB1 construct (Fig. 7B). Pericellular ATP was detected in TSA cells after transfection with the pGen2.1 vector and a DNA construct that expresses a cell membrane targeted firefly luciferase fused to a folate receptor N-terminal leader sequence and a C-terminal GPI anchor (Fig. 7C). ATP detection was validated by measuring the luminescence from the plasma membrane luciferase (pmeLUC) in the presence of ATP, $MgSO_4$, and luciferin substrates.

Results: Cell lines to detect each arm of ICD were established and validated. To understand the kinetics of CRT redistribution, and release of HMGB1 and ATP, each cell line was exposed to IR at various times, doses, and fractionation schedules.

Conclusions: Genetically engineered cell lines can report the activities of CRT, HMGB1, and ATP. Future studies include screening for IR based regimens that potently stimulate all three arms of ICD with the potential for clinical translation.

Key Words: Breast cancer, Immunogenic cell death, Abscopal.

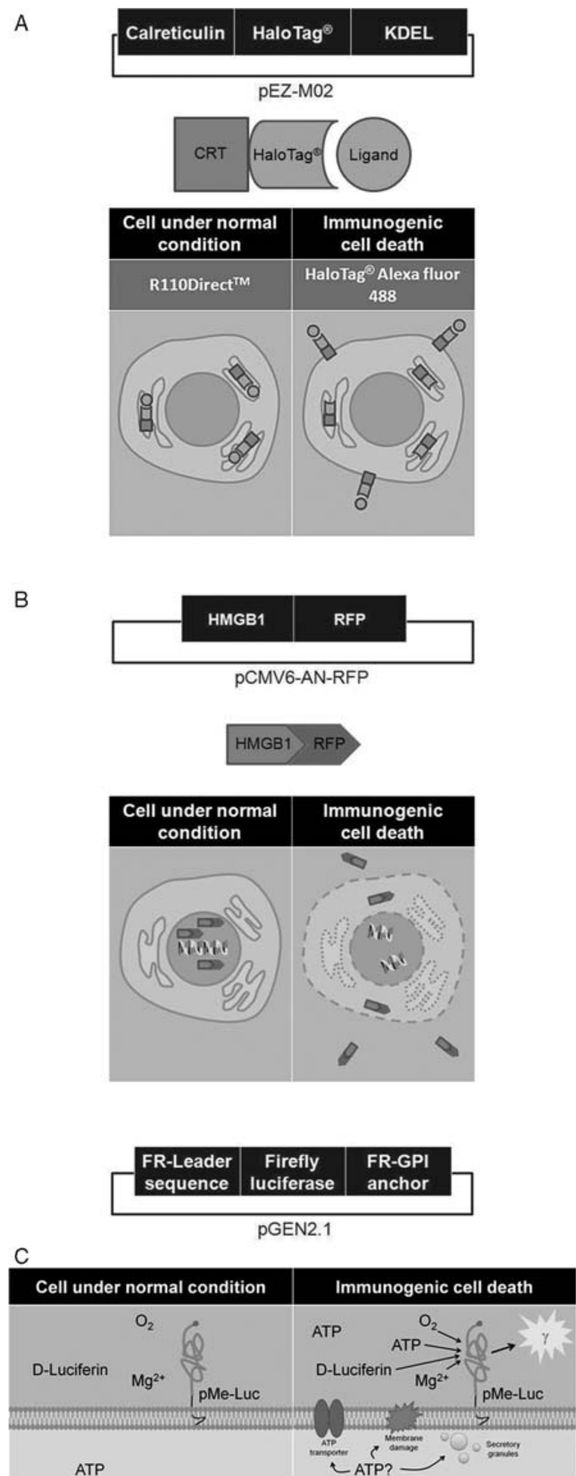

FIGURE 7. Immunogenic cell death assay for radioimmunogenicity.

Rapid Assessment of Time, Dose, and Fraction Dependent Release of Extracellular HMGB1 After Ionizing Radiation (IR)

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Purpose: Immunogenic cell death (ICD) induced by standard therapies is described by Kroemer and Zitvogel as being orchestrated through three indispensible pathways: 1) surface translocation of calreticulin and extracellular release of 2) high-mobility group protein B1 (HMGB1) and 3) ATP. HMGB1 promotes inflammation upon extracellular release of dying tumor cells. We applied an assay from engineered TSA cells (a BALB/c murine mammary cancer) to rapidly measure HMGB1 release in response to IR. Methods: To investigate HMGB1 release kinetics, TSA cells were transfected with a pCMV6-AC-GFP or pCMV6-AN-RFP expression vectors containing an HMGB1 construct (Fig. 8A). HMGB1 nuclear localization was confirmed via fluorescent microscopy (Fig. 8B). Cells were seeded in 6-well plates and treated with IR. Media was collected and transferred to 96-well plates for extracellular HMGB1 spectrophotometric analysis.

Results: The fold increases (+ /-SD) of HMGB1-GFP were detected in the media at various times, doses, and fractionations. Controls were standardized to 1.0. The fold increase in HMBGB1-GFP release at 0, 24, 48, and 72 hours in untreated cells were 1.0 + /-0.04, 1.05 + /-0.03, 1.11 + /-0.06, and 1.23 + /-0.05, respectively. In cells treated with IR 20 Gy, the fold increases at 0, 24, 48, and 72 hrs were 0.92 + /-0.02, 0.96 + /-0.04, 1.42 + /-0.04, and 1.85 + /-0.07, respectively (Fig. 8C). The fold increases of HMGB1-GFP release after 72 hours of treatment with IR 0, 2, 10, and 20 Gy were 1.0 + /-0.08, 1.12 + /-0.11, 1.47 + /-0.09, and 1.54 + /-0.11, respectively (Fig. 8D). Finally, the fold increases of HMGB1-GFP release in untreated cells, cells exposed to 20 Gy x1, and 8 Gy x3 were 1.0 + /-0.11, 1.45 + /-0.07, and 1.41 + /-0.06, respectively (Fig. 8E).

Conclusions: HMGB1 transfected cells permit the rapid assessment of HMGB1 release in response to IR in a time, dose, and fraction

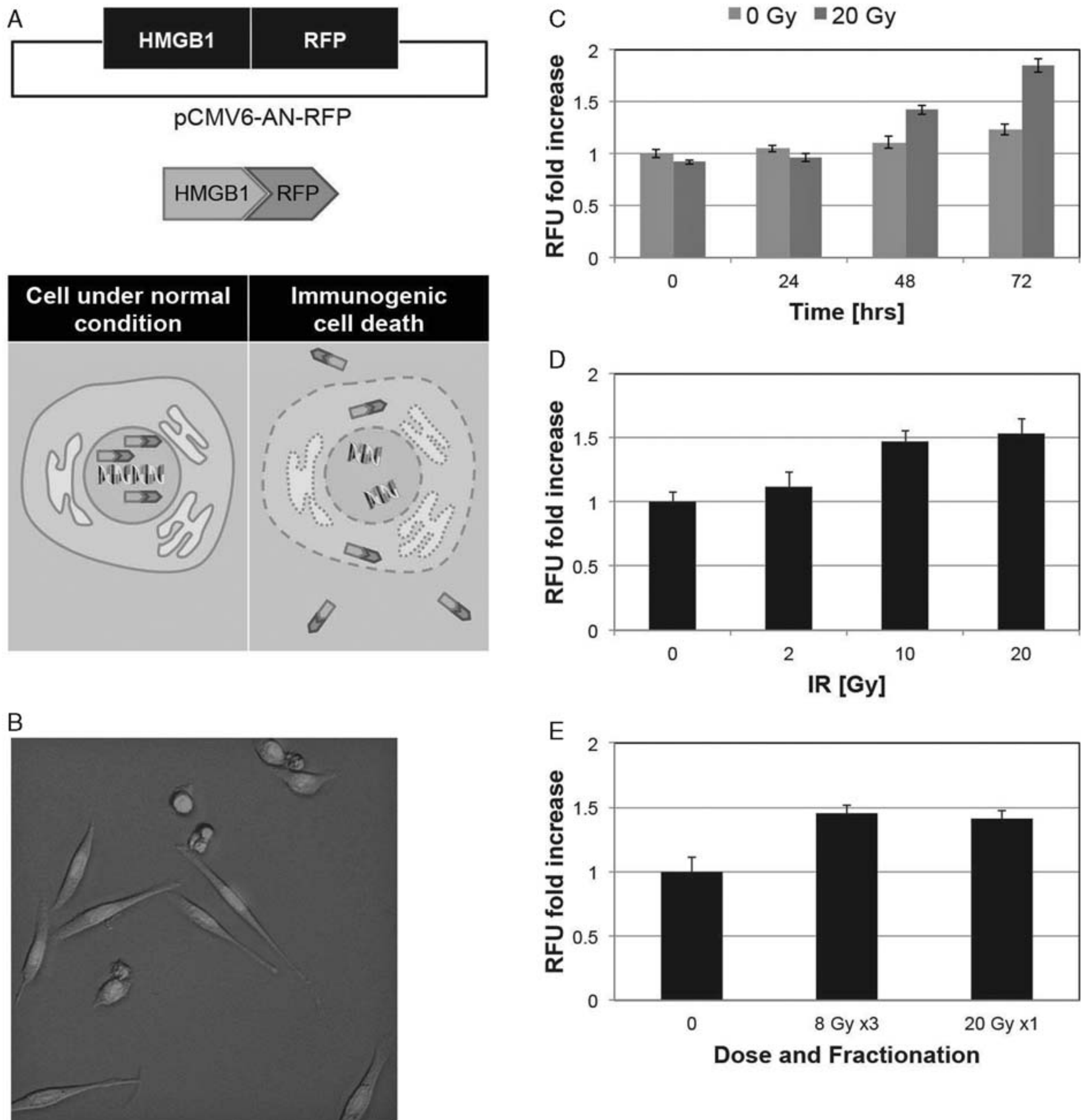

FIGURE 8. HMGB1 release after IR exposure.

dependent manner. Future studies include screening for IR based regimens that potently stimulate ICD.

Key Words: Breast cancer, Immunogenic cell death, Abscopal.

Rnadjuvant® Combined With Antigen Induces Superior Anti-tumor Activity Compared to Poly I:C and has a Very Favourable Safety Profile

Regina Heidenreich, Mariola Fotin-Mleczek, Patrick Baumhof, Birgit Scheel, Söhnke Voss, Thomas Kramps, Karl Josef Kallen. *CureVac GmbH, Tübingen, Germany.*

Most peptide or protein based tumor antigens are weakly immunogenic. Strong adjuvants with good safety profiles are thus required to induce potent and persistent immune responses against cancer. RNAdjuvant® is an adjuvant based on a non-coding RNA developed by us, which is protected against degradation and can be easily combined with relevant antigens.

RNAdjuvant® leads to a strong activation of antigen presenting cells in in vitro studies on human peripheral blood monocytes with increased expression of activation markers and secretion of TH1-type cytokines. In vivo, vaccination with RNAdjuvanted antigens induces balanced immune responses, comprising humoral IgG1 and IgG2a responses as well as induction of antigen-specific effector T-cells and, importantly, memory T-cells.

Combination of RNAdjuvant® with both, ovalbumin or the ovalbumin-derived SIINFEKL epitope, triggers a strong antigen-specific cytotoxic T-cell response that is barely observed after vaccination with ovalbumin or SIINFEKL alone. Only the response induced by vaccination with the RNAdjuvant®/

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ovalbumin combination translated into potent prophylactic and therapeutic anti-tumor activity, whereas the T-cells elicited against SIINFEKL were irrelevant for the anti-tumor effect.
RNAdjuvant® also induced strongly enhanced cytotoxic T-cell responses against a long-chain peptide of the human papillomavirus (HPV)-16 derived E7 protein compared to the standard adjuvant PolyI:C. Immunization with RNAdjuvanted E7 peptide largely protected mice against challenge with the tumor model cell line TC-1. Remarkably, RNAdjuvanted® E7 peptide showed a much superior anti-tumor activity in a therapeutic TC-1 model compared to Poly I:C. RNAdjuvant® even impairs the growth of extremely large tumors in this model. A toxicology study performed with a relevant tumor antigen revealed no conspicuous findings. Our data suggest that RNAdjuvant® has an extremely favourable risk/benefit profile that could help to propel forward the field of cancer vaccines.
Key Words: Cancer immunotherapy, Adjuvant, HPV.

IMCMAGE1: A Novel BI-specific Biologic Re-directing T Cells to Kill MAGE-A3/A6 Presenting Cancers

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In a minority of cancer patients immunotherapy has shown the capacity to eradicate tumours leading to clinical remission and the promise of a cure. In the majority of patients however, a cure remains elusive due to immune evasion by cancers; HLA-down-regulation and immunosuppression are two mechanisms adopted by cancers to promote their survival and proliferation. To overcome these challenges we have developed bi-specific soluble biologics termed ImmTACs (Immune mobilising mTCR against cancer) to re-direct the immune system to recognise and kill cancers.
Antigenic peptide fragments presented by HLA molecules on the surface of cancer cells constitute the largest class of cancer associated targets. T cells scan the HLA-peptide (pHLA) antigens being presented; sufficient recognition by the harboured T Cell Receptor (TCR) will result in T cell activation and killing of the antigen presenting cell. In cancer patients this process is inefficient partly due to the low affinity TCRs expressed by tumour specific T cells and the low abundance of pHLA on cancers. ImmTACs comprise a soluble TCR with an enhanced affinity for cancer associated pHLA (targeting end) fused to an anti-CD3 scFv (effector end), enabling potent T cell re-direction. Our pipeline constitutes a number of ImmTACs targeting various antigen pHLA complexes relevant to numerous cancer indications.
IMCmage1 is a novel ImmTAC targeting MAGE-A3168-176 in the context of HLA-A1. MAGE-A3 is a well validated cancer testis antigen expressed in a variety of cancers including myeloma, NSCLC, prostate cancer, melanoma, bladder cancer, oesophageal cancer and others. IMCmage1 re-directs T cells from cancer patients or healthy donors to kill a range of MAGE positive cell-lines in vitro; this activity is observed against cells presenting as few as 40 epitopes per cell and is coupled with the release of pro-inflammatory cytokines including IFNγ, TNFα, IL-2, and MIP1β. We also demonstrate that IMCmage1 specifically targets and kills the myeloma associated population within CD138 + cells extracted from the marrow of a stage III myeloma patient. IMCmage1 activity is not influenced by the presence of bone stromal cells, which are known to maintain survival of myeloma cells. IMCmage1 specificity was confirmed by exposure to a panel of HLA-A1 MAGE negative primary cells derived from various organs including heart, skin, lung and others; no significant activity was detected. A phase I clinical trial in multiple myeloma to assess tolerability and establish a maximum tolerated dose is planned to commence in 2012.
Key Words: TCR, Immunomodulation, Targeted therapeutics.

Antibody-directed CPG Targets Tumor Microenvironment and Provides Active Immunotherapy

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An obstacle in the immunotherapy of cancer is the cancer's ability to escape detection by the immune system. Immune adjuvants, such as toll-like receptor (TLR) agonists, delivered to the tumor microenvironment may be able to block this escape by activating an innate response, which can lead to a subsequent adaptive response. Unmethylated CpG oligodeoxynucleotides (ODNs) are TLR-9 agonists known to stimulate dendritic cells and have been used primarily as an adjuvant injected locally at the site of interest. However, for systemic diseases such as cancer, the local administration of CpG would limit its potential to the site of injection. To address this, we have chemically linked active CpG motif analogues to chTNT-3, a monoclonal antibody which targets tumor necrosis, a site rich in tumor antigens. This approach to cancer immunotherapy is novel in concept and may yield important information regarding the effectiveness of innate immunity in the treatment of cancer. Our findings validate the usefulness of this approach and show that, compared with the parental antibody, the CpG immunoconjugate is able to target tumor and produce a 50% greater reduction in tumor growth. We are currently addressing the mechanism for this observation by characterizing the tumor-infiltrating immune cells in CpG-treated, tumor-bearing mice. It is anticipated that the antibody-directed CpG will enable research to optimize the effectiveness of CpG, as well as other TLR-agonists, especially in combination with other forms of immunotherapy, for the successful treatment of solid tumors.
Key Words: Adjuvant, Dendritic cell, Targeted therapeutics.

Analysis of PSA-specific T Cell Response in PSA-transgenic Mouse: A Useful Model to Study Prostate Cancer Immunotherapy

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The idea of manipulating immune system for cancer treatment represents one of the most valuable approaches. However, the development of immunotherapy approach for the treatment of human cancer using cancer-specific antigens is dependent on the ability to overcome immune tolerance to the antigen. While various approaches are in developmental stages, the use of adenovirus vector (Ad) as an immunotherapy agent to deliver tumor-associated antigens is an attractive and versatile vector system. In this study we tested the utility of Ad-vector-based prostate cancer vaccine inducing anti-PSA T cell response in PSA-tg (prostate-specific antigen-transgenic) mouse model. PSA-tg mouse expresses human PSA in the prostate, analyzed at the mRNA (RT-PCR) as well as protein (immunohistochemical staining) levels. The overall intensity and the amount of PSA secretion is associated with increasing age (higher in 15-month-old mice), and the secretion of PSA is confined to the lumen. To analyze the ability of Ad-vector-based prostate cancer vaccine (Ad/PSA + PSCA) inducing PSA-specific T cell response, PSA-tg mice were immunized subcutaneously in the collagen matrix. Single immunization with Ad-vaccine (108pfu) expressing prostate-specific antigens resulted PSA-specific T cell response almost parallel to Ad-LacZ control immunization. However, homologous prime-boost immunizations at an interval of three weeks apart induces strong anti-PSA T cell response as illustrated by ELISPOT assay and intracellular cytokine staining for IFN-gamma by CD8 + T cells, and ELISA assay for IFN-gamma. These observations suggest that the use of collagen matrix with Ad-vector-based vaccine induces strong immune T cell response against the self-antigen as well as circumvent the pre-existing anti-Ad immunity. This finding is important in that most humans have pre-existing levels of anti-adenovirus antibodies due to prior natural exposure to the virus. Thus, PSA-tg mouse may represent a useful model to understand the mechanistic regulation of immune response against the self-antigen to break immunological tolerance.

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Key Words: Cancer immunotherapy, Active immunotherapy, Animal model.

Pre-existing Immunity to Cancer Over-expressed Antigens Inversely Correlate With the Level of Myeloid Suppressor Cells in Circulation

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Cancer immunotherapy remains a promising approach in cancer therapeutics. Immunogenicity of tumors is highly variable and it can be challenging to determine why robust immune responses fail to be induced in vivo, despite recognition of tumor-associated/over-expressed antigens (TAA). The extent of immune induction may depend on the balance between immunogenicity of TAA and the immunosuppressive mechanism(s) at play at different stages of cancer progression. We have developed two therapeutic cancer vaccines incorporating peptides derived from TAAs; DPX-0907 containing seven antigens designed for breast, ovarian and prostate cancers and DPX-Survivac targeting the survivin protein which is over-expressed in several cancer types. The present study examines the pre-existing immunity to these 8 TAA in metastatic cancer patients not on concurrent chemotherapy by immunofluorescence using MHC-multimers that bind TAA-specific CD8 T cells and attempts to correlate it with myeloid suppressor cells (MDSC) in blood. Among the 44 HLA-A2 positive patients studied, samples from 7/14 (50%) breast, 6/15 (40%) ovarian and 2/15 (13%) prostate cancer patients showed antigen-specific CD8 T cells to at least one of the TAA. Ovarian cancer patients had T cells against 6 of 8 TAA, representing the cohort with the broadest immune response, while breast and prostate cancer patients had specific T cells for 3 of 8 TAA. For the entire cohort (n = 44), 18% (n = 8) of patients had pre-existing immunity to at least one TAA, 14% (n = 6) to 2 TAA and 2% (n = 1) to ≥ 3 TAA. Interestingly, three of the TAA (TACE, EDDR1 and survivin) had more frequently detectable specific T cells than other TAA. Frequency of CD11b + CD33 + HLA-DR- MDSC among all gated leukocytes was significantly higher in prostate cancer patients (0.83 ± 0.19. $P < 0.01$) compared to breast/ovarian cancer patients (0.2 ± 0.05/0.48 ± 0.1). The absolute MDSC count in the peripheral blood was also significantly higher in these patients compared to other two cancer types. Our findings suggest that patients with metastatic prostate cancer may have higher levels of circulating immunosuppressive MDSC hindering the establishment of anti-cancer immunity. A lower frequency of MDSC was observed in patients who had TAA-specific immunity compared to those without detectable immunity suggesting that patient selection for immunotherapy may be optimized by targeting those where specific vaccines may boost pre-existing immunity in conditions of less severe immunosuppression.

Key Words: Cancer vaccine, MDSC, CD8 + T cells.

Increased CD4 T Cell and Antibody Responses by Addition of Recombinant HER2 Protein to MVA-BN®-HER2

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BN ImmunoTherapeutics (BNIT) specializes in developing novel active immunotherapies for cancer. These therapies use recombinant poxviruses engineered to express tumor associated antigens (TAAs), with the intent of generating effective immune responses against the patients' cancer. MVA-BN®-HER2, is in Phase I clinical trials for the treatment of HER-2-positive breast cancer. This immunotherapy is derived from a clonal isolate of the highly attenuated Modified Vaccinia Ankara (MVA) virus stock known as MVA-BN®. The attenuated phenotype of MVA-BN® provides additional safety when given to immunocompromised patients, while providing a strong adjuvant activity to transgenic antigens that triggers adaptive and innate immunity. MVA-BN®-HER2 expresses a modified form of human epidermal growth factor receptor 2 (HER-2) that includes two universal T cell epitopes from tetanus toxin to facilitate the induction of effective immune responses against HER-2. Our Phase I clinical results show that HER-2-specific antibody and T cell responses were induced in patients treated with MVA-BN®-HER2. Previous preclinical studies showed that anti-tumor activity of MVA-BN®-HER2 was characterized by the induction of Th1-biased antigen-specific immune responses in preclinical HER-2-specific tumor models. Tumor efficacy was accompanied by increased infiltration of tumors with highly activated, HER-2-specific T cells and a decrease in the frequency of regulatory T cells (Treg) (Mandl et al, CII, 2012 Jan; 61(1):19-29).
To further characterize the mechanism of action of MVA-BN®-HER2 the immunologic function of the MVA-BN® vector as an adjuvant was explored. Here we describe preclinical experiments comparing the immune responses and anti-tumor efficacy of MVA-BN® or MVA-BN®-HER2 as adjuvants when mixed with recombinant HER2 protein. Our data demonstrate that MVA-BN® has potent adjuvant activity and requires live virus. HER-2 specific immune responses and anti-tumor efficacy were induced; however, expressing the HER2 protein directly from the vector as in MVA-BN®-HER2 was superior. Addition of protein to MVA-BN®-HER2 further increased HER-2 specific immune responses particularly with respect to CD4 T cell and antibody responses. This resulted in improved anti-tumor efficacy in the TUBO breast cancer model in which anti-tumor efficacy has been described as being antibody dependent. These data show that the anti-tumor activity of MVA-BN®-HER2 could potentially be increased by adding HER2 protein to the recombinant vector.
S.J. Mandl and R.B. Rountree contributed equally to this work.
Key Words: Cancer immunotherapy, Adjuvant, Animal model.

The Cancer/Testis Antigens Ropporin and AKAP-4 are Novel Targets for Multiple Myeloma Immunotherapy

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Multiple myeloma (MM) is an incurable malignancy caused by malignant plasma cells accumulating in the bone marrow. Despite recent improvements in standard pharmacologic treatments of MM, immunotherapy may prove to be more effective due to its higher specificity and lower toxicity. Ropporin and AKAP-4 are testis-specific proteins localized in the sperm flagella. Comparing Ropporin and AKAP-4 expression in healthy and MM samples, we did not detect expression in the normal tissues, but positive signals were found in the majority of the MM primary samples. Ropporin/AKAP-4 immunogenicity was confirmed by the presence of specific antibodies detected by enzyme-linked immunosorbent assay in patients' sera. We suggest that Ropporin and AKAP-4 are promising targets for MM immunotherapy, as we were able to generate Ropporin and AKAP-4-specific human leukocyte antigen class I-restricted cytotoxic lymphocytes able to kill autologous MM cells.

Key Words: Active immunotherapy, Multiple myeloma, Tumor associated antigen.

Active Immunotherapy With Prostvac® Demonstrates Potent Anti-tumor Efficacy in a Mouse Model of Prostate Cancer

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BN ImmunoTherapeutics (BNIT) specializes in developing novel active immunotherapies for cancer. These therapies use recombinant poxviruses engineered to express tumor-associated antigens (TAAs), with the intent of generating effective immune responses against the patients' cancer. PROSTVAC® is a candidate product for the treatment of prostate cancer for which a global Phase III clinical trial (PROSPECT) was recently initiated. This product is composed of two different viral vectors derived from a recombinant vaccinia virus (PROSTVAC™-V) and a recombinant fowlpox virus (PROSTVAC™-F). Both vectors contain transgenes encoding prostate-specific antigen (PSA) and a triad of costimulatory molecules (B7-1, ICAM-1, and LFA-3), designated as TRICOM™. Patients are immunized using a prime-boost strategy consisting of an initial treatment with PROSTVAC™-V followed by repeated boosting with PROSTVAC™-F to maximize the immune responses against the PSA tumor-antigen. Here we show preclinical data characterizing PROSTVAC® activity in mice. Treatment with either PROSTVAC™-V or PROSTVAC™-F induced PSA-specific antibody and T cell responses; however, PSA-specific responses were further increased by the prime/boost strategy, particularly with respect to the frequency of responding CD8 T cells. These CD8 T cells produced IFN-gamma and degranulated in an antigen-specific manner. Furthermore, PROSTVAC® treatment resulted in strong efficacy in a mouse model of prostate cancer. In this model, treatment with PROSTVAC resulted in anti-tumor efficacy accompanied by a Th1-biased response against PSA. In contrast, growth of tumors in control mice induced only non-protective PSA-specific responses with strong Th2 bias. To improve anti-tumor efficacy in established tumors, combination therapy with anti-CTLA-4 blockade was also explored.
Overall, these animal studies help define the activity and mechanism of action of PROSTVAC® which is currently being evaluated in the clinic.
S.J. Mandl and R.B. Rountree contributed equally to this work.
Key Words: Cancer immunotherapy, Animal model, Prostate cancer.

The Utilization of Pseudomonas Aeruginosa Exotoxin T as a Potential Chemotherapeutic Agent for Solid Tumors

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Despite significant advancements in the treatment of metastatic melanoma, approved therapies fall short of providing long-term control, especially in patients with advanced disease, which are associated with 5-year survival rates of 10-15%. Therapeutic agents are needed to induce potent cell death by targeting multiple cellular pathways and elicit a robust anticancer immune response. Pseudomonas aeruginosa exotoxin T (ExoT) is known to kill with different modalities of cytotoxicity and induce a proinflammatory environment, thus we hypothesized that this exotoxin could be an ideal therapy for melanoma and other malignancies. To demonstrate that this toxin is effective at killing melanomas and other carcinoma lines, we co-cultured cancer cells with ExoT-expressing Pseudomonas aeruginosa. Compared with the chemoreagent cisplatin, ExoT induced more potent cytotoxicity and faster kinetics of killing in B16 melanoma cells. To determine if ExoT was sufficient to kill melanoma, we delivered this bacterial exotoxin to B16 cells via transient transfection of a plasmid containing a direct fusion of EGFP to ExoT. We examined the extent of ExoT-specific killing in the context of transfection efficiency by flow cytometry and by time-lapse video microscopy. Finally, to further examine the potential of ExoT as a valuable therapeutic in the treatment of solid tumors, we examined its cytotoxic effects in vivo by delivering ExoT to B16 melanoma tumors in mice. We found that this toxin was able to induce potent cytotoxicity in a wide variety of cancer cell lines, demonstrating enhanced killing compared with known chemotherapeutic drugs, like cisplatin. Together, our data suggests that P. aeruginosa ExoT may be an effective new therapy for treating not only melanoma, but also many different solid-tumor malignancies. Our future studies will include a detailed analysis of exotoxin-mediated immunogenicity in cancer.
Key Words: Immunogenic cell death, Chemotherapy, Melanoma.

Induction of Multipotent Vδ2-negative γδT-cells After CMV-reactivation in Allogeneic Stem Cell Transplantation

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Human cytomegalovirus (CMV) infections and relapse of disease remain major problems after allogeneic stem cell transplantation (allo-SCT), in particular in combination with CMV-negative donors or cordblood-transplantations. Expansion of Vδ2-negative γδT-cells after CMV-infection in healthy individuals and after transplantation has been reported, and provides great promise as therapeutic tool. However, the contribution of distinct γδT-cell-subsets expanding during CMV-infection, including specificity and molecular interaction with their target remains unclear. We report, contrary to previous observations, that γδT-cell expansions after CMV-infections during allo-SCT with conventional and cordblood-donors precede expansions of αβT-cells, and that elicited γδT-cells have diverse functions: they react not only to CMV-infected fibroblasts but also primary leukemic blasts, and mediate maturation of dendritic cells (DCs). CMV- and leukemia-reactivity were restricted to the same clonal population, whereas other Vδ2neg T-cells had DC-maturing capacities. Moreover, Vδ2neg-TCRs mediated DC-maturation and leukemia-reactivity, but surprisingly not CMV-reactivity. Finally, signalling through selected leukemia-reactive γδTCRs depended on CD8αα, demonstrating a co-stimulatory role of human CD8αα for distinct γδTCRs. In summary, our data support a so far underestimated diverse role of γδT-cells elicited during CMV-reactivation in shaping an immune response, either directly by attacking CMV-infected cells and leukemic blasts or indirectly by facilitating adaptive immune responses.
Key Words: T cells, Innate immunity, Leukemia.

Successful Treatment of Established Mouse Melanoma With IL-12 Electrotransfer is Dependent on the Delivery Parameters Used

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Electrotransfer (ET), a reliable physical method of delivering plasmid DNA (pDNA) directly to tumors, has been used in a number of clinical trials for melanoma, squamous cell carcinoma and basal cell carcinoma. ET of interleukin 12 (IL-12) directly to tumors has been shown to generate a local and systemic anti-tumor effect in both preclinical and clinical studies. It is important to achieve the appropriate balance between transgene expression and tissue damage in order to stimulate the host immune response to reach the clinically desired outcome. Here we examine the effects of varying the ET parameters, electrodes and how the resultant expression levels of pDNA influences the outcome of IL-12 ET therapy.
Plasmid DNA was injected into established tumors of C57BL/6J mice and electric pulses applied. This was done a total of three times on days 0, 4 and 7 to complete the treatment protocol. The plasmids used were an empty vector control (pUMVC3), a low expresser (pUMVC3-mIL12) and a higher expresser (pAG250-mIL12) of murine IL-12. A caliper applicator consisting of two metal plates or a circular applicator comprised of six penetrating electrodes was used

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to deliver the pulses. The pulses applied were either high voltage, short duration (HVSD) or low voltage, long duration (LDLV). These conditions generate electric fields that mediate different efficiencies of gene transfer. The tumor volumes were measured for nine weeks after which the surviving mice were challenged by subcutaneous injection of B16.F10 melanoma cells on the opposite flank. Gene expression was measured by ELISA after a single treatment. Tissue sections were collected for histology at 24 hours after a single treatment. At least 89 percent of the mice treated with ET and pUMVC3-mIL12 showed tumor regression and were visibly tumor free at the end of nine weeks. This was not the case with mice treated with ET and pAG250-mIL12. Mice treated with pUMVC3-mIL12 and HVSD pulses had the highest survival rates of all the treated groups. They also had the lowest levels of IL-12 expression. H& E staining revealed more damage to tumors treated with LVLD pulses than HVSD pulses. Tumor infiltrating lymphocytes were present in most of the ET treated tumors but there were more CD4 + and CD8a + cells in the tumors treated with pUMVC3-mIL12. These results indicate low levels of IL-12 expression in tumors treated with pIL-12 and ET are best for generating local and systemic anti-tumor response that correspond with a more successful outcome. This finding is important in order to improve ET-based therapies for melanoma patients.

Key Words: IL-12, Melanoma, Immunotherapy.

Differential Effects of the Tyrosine Kinase Inhibitors on T Cell Growth Properties and Activity

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Tyrosine kinase inhibitors (TKI) have been successfully implemented as first-line therapy for the treatment of malignant tumors, including renal cell carcinoma (RCC). Treatment of RCC with TKI results in significant objective clinical responses and a longer progression-free survival of patients by the inhibition of cell growth, angiogenesis and the induction of apoptosis. There is also evidence that different TKI are able to modulate the immune response. Concerning sorafenib and sunitinib, effects on the frequency and function of T cell subpopulations, DCs as well as MDSC have been reported. Thus, for the optimized clinical use of these inhibitors, a better understanding of their effects on the anti-tumor specific immune response as well as against opportunistic infections is required. Although immunomodulatory effects of sunitinib and sorafenib have been reported, little is yet known about axitinib. Therefore, T cells obtained from malignant hematopoietic cells as well as peripheral blood lymphocytes from healthy donors were exposed to different TKI to monitor the effect on the growth properties and the induction of apoptosis. All three TKI (sunitinib, sorafenib and axitinib) dramatically reduced the T cell proliferation rate, which was at least partially associated with an induction of apoptosis as determined by an altered annexin V expression, caspase activity as well as disruption of the mitochondrial potential. In contrast to sunitinib or sorafenib, the axitinib-mediated growth inhibition was biphasic in Jurkat cells, and hardly no effect of axitinib on the viability of stimulated PBMC was detected. Cell cycle arrest in the G2/M phase could only be detected in the presence of axitinib, but not for sunitinib or sorafenib. Based on the comparative analysis of apoptosis induction in Jurkat cells, these substances exhibit distinct apoptotic mechanisms.

Whereas treatment with axitinib resulted in a slight up-regulation of the early activation marker CD69 in Jurkat cells, treatment with sunitinib or sorafenib led to a strong down-regulation of CD69. In addition, TKI-induced alterations within the protein expression profiles of activated and unstimulated Jurkat cells in response to TKI-treatment were further characterized by 2D-based proteomic analysis. So far, Jurkat cells revealed > 20 differentially expressed protein spots. Functional analysis of TKI-regulated proteins are still ongoing, but will likely shed light into the biologic activity of these TKI in T cells.

Key Words: Targeted therapeutics.

Development of Bivalent *Listeria Monocytogenes* -LLO Immunotherapy That Concomitantly Targets Tumor Cells and Angiogenesis

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Numerous published reports show that recombinant *Listeria monocytogenes* (*Lm*-LLO)-based immunotherapy expressing either tumor associated antigens (TAA) or angiogenesis associated antigens fused to an immunogenic fragment of listeriolysin O (LLO) demonstrate therapeutic efficacy in different mouse tumor models such as lung, breast, prostate or melanoma. Overexpression of tumor associated antigens (TAA) such as HER2/neu and high molecular weight melanoma associated antigen (HMW-MAA) are associated with aggressive high-grade tumors leading to disease progression and reduced survival. HMW-MAA has been reported as a TAA in triple negative breast tumors and is also expressed at high levels both by activated- and tumor angiogenic-pericytes associated with neovascularization *in vivo*. The *Lm*-LLO-cHER2 immunotherapy developed using a chimeric HER2/neu (cHER2) was found to regress tumors, elicit a strong T cell immune response and break immune tolerance towards the HER2/neu self-antigen in experimental animals. The *Lm*-LLO-HMW-MAA immunotherapy has been shown to eradicate established breast tumors, reduce microvascular density and protect against tumor recurrence. Therefore, we hypothesized that bivalent *Lm*-LLO immunotherapy capable of delivering two different antigens would likely have a synergistic effect on decreasing tumor growth by targeting two independent mechanisms that support tumor growth; 1) tumor angiogenesis, and 2) tumor cell surface marker, thus improving the therapeutic efficacy of the agent. In addition, a bivalent construct creates a flexible platform for future use. A bivalent *Lm*-LLO immunotherapy (BV-168) was created that expresses and secretes both the cHER2 and HMW-MAA antigens as LLO-based proteins and is based on a highly attenuated strain *Lm* Δ *dal dat actA*, which is cleared 48 hours post-injection in wild type- and interferon gamma knockout-mice. Initial characterization of the BV-168 indicates that the two antigens cHER2 and HMW-MAA are stably expressed and secreted after two *in vivo* mouse passages. Currently, we are evaluating the anti-tumor effects and antigen specific immune responses generated by BV-168 in both transplantable and transgenic mouse models. If successful, BV-168 may offer a new immunotherapy for the treatment of HER2 overexpressing cancers including breast, GI, CNS and others.

Key Words: Breast cancer, Cancer vaccine, Immunotherapy.

Pseudomonas Aeruginosa Exotoxin T Induces Cytotoxicity and Blocks Apoptotic Compensatory Proliferation Signaling

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Most cancer therapies induce apoptosis in cancer cells, however, tumors frequently become resistant to therapy. While resistance can result from many different factors, one mechanism that has not been well studied is the apoptotic compensatory proliferation. For several decades it has been postulated that dying cells can induce compensatory proliferation in neighboring cells to maintain tissue homeostasis. The ability of dying cells to induce compensatory proliferation could limit the effectiveness of cancer therapies that induce apoptosis, however, the molecular components of compensatory proliferation have remained unknown. We have previously shown that the Pseudomonas aeruginosa virulence factor Exotoxin T causes potent apoptosis in HeLa cells. During the investigation of how ExoT induces cytotoxicity we identified the adapter protein Crk as a component of apoptotic compensatory proliferation. We have found that apoptotic cells, prior to their demise, form and release specialized complexes, which induce proliferation in bystander cells upon contact. We refer to these complexes as apoptotic compensatory proliferation complexes (ACPC). Importantly, ExoT targets Crk for ADP-ribosylation, which blocks apoptotic compensatory proliferation signaling while

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inducing potent apoptosis. This finding indicates that apoptotic compensatory proliferation signaling and apoptotic programmed cell death are distinct cellular processes which can be uncoupled from each other. We propose that the induction of apoptotic compensatory proliferation is one of the main mechanisms for tumor resistance to cancer therapy. Further understanding of the compensatory proliferation pathway could greatly enhance our knowledge of cancer biology. ExoT is capable of inducing immunogenic cytotoxicity and also uncouples apoptotic compensatory proliferation signaling from apoptotic cell death. Therefore, we believe ExoT could be used as a promising new therapy for cancer.
Key Words: Apoptosis, Chemotherapy, Tumor microenvironment.

The Anti-tumor T Cell Response Plays a Critical Role in the Therapeutic Effect of Dasatinib on C-kit Mutant Mastocytoma and can be Potentiated by Anti-OX40 Antibody

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The therapeutic effects of molecular targeted drugs are believed to be primarily dependent on direct effect on tumor cells. However, using a c-kit mutant mastocytoma model P815, we show that the underlying T cell-mediated anti-tumor immunity contributes substantially to the therapeutic effect of a c-kit inhibitor dasatinib, and this therapeutic effect can be potentiated by combining with a costimulatory antibody anti-OX40. We observed that 3 days of dasatinib treatment significantly decreased the tumor volumes and slightly prolonged the survival of the mice. However, depletion of CD4 + or CD8 + T cells effectively abrogated the anti-tumor effect and survival benefit provided by dasatinib, suggesting that the therapeutic effect of dasatinib on P815 is crucially dependent on the presence of a T cell-mediated anti-tumor immune response. P1A tetramer staining and IFN-γ intracellular staining of PBMC showed that dasatinib treatment significantly enhanced the tumor antigen-specific CTL response. Since we also found that 3 days of dasatinib treatment augmented CD8 + T cell response in a tumor-free vaccine model, we speculated that the enhanced effector T cell response might be caused by decreased levels of Treg cells after dasatinib treatment and direct inhibitory effect of dasatinib on Treg function. Addition of anti-OX40 antibody further improved the therapeutic effect of dasatinib resulting in the cure of most mice. Flow cytometry analysis of tumor-infiltrating lymphocytes showed that anti-OX40 alone enhanced the overall infiltration of CD8 + effector T cells but not the infiltration of tumor-specific T cells. While, with dasatinib increasing tumor-specific CTL level in circulation, the combined regimen led to significantly increased intratumoral infiltration of tumor-specific CTL and more robust therapeutic effect. Realtime PCR showed that this combination significantly up-regulated the IFN-γ-induced Th1 chemokines CXCL9,10 and 11 in the tumor microenvironment, suggesting that combining anti-OX40 with dasatinib leads to the formation of a positive feed-back loop composed of CTL,IFN-γ and Th1 chemokines in situ. This study shows that the development of anti-tumor immune response is an important underlying contributory factor to the therapeutic effect of targeted therapy and describes a complementary mechanism by which molecular targeted drug and immune-boosting antibody could be combined to improve anti-tumor efficacy.
Key Words: Dasatinib, Immunomodulation, Tumor microenvironment.

TARGETING IMMUNE SUPPRESSION

Modulation of Regulatory T Cells by Targeting The NFAT-FOXP3 Protein:Protein Interaction

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Cancer vaccines often generate elevated numbers of tumour-specific T-cells however these are generally insufficient to control disease. Considerable evidence suggests that CD4 + CD25 + regulatory T-cells (Treg) are largely responsible for preventing effective anti-tumour immune responses. Thus the development of novel strategies to manipulate the suppressive activity of Treg remains an important goal for cancer immunotherapy. Agents targeting Treg in the clinic have shown variable efficacy and considerable toxicity, e.g. the use of anti-CTLA4 antibodies has achieved some significant successes in clinical trials for several malignancies. Although effective, these antibodies have a relatively long clearance time which is thought to promote aggressive autoimmune responses.

CD4 + CD25 + Treg cells are characterized by the transcription factor FOXP3 which is a master regulator of the function and development of Tregs. FOXP3 functions through the obligatory interaction with another transcription factor NFAT (nuclear factor of activated T cells) resulting in repression of cytokine gene expression as well as the activation of the Treg marker genes CTLA4 and CD25. We have taken a novel approach to targeting Treg by developing a peptide (HWFT) that disrupts the interaction between the Treg specific transcription factor, FOXP3 and its obligatory co-factor NFAT. In mice HWFT triggers apoptosis specifically in Treg in vitro, whilst in humans it inhibits their suppressive capacity without killing. At the molecular level we have shown that the DNA binding ability of FOXP3 is abolished in the presence of HWFT. In order to evaluate the effect of HWFT treatment in mouse tumour models, the CD4 + CD25 + subset in peripheral blood, spleen lymphocytes and tumour-infiltrating lymphocytes from HWFT-treated compared to untreated CT26 colon-carcinoma-bearing BALB/c mice will be analyzed by flow cytometry. The findings from these animal experiments will also be presented. This novel approach of targeting the FOXP3/NFAT complex may provide an additional strategy for abrogating local immune suppression in tumours exerted by Treg.
Key Words: Immunosuppression, Regulatory T cells.

Driving Anti-cancer Immune Responses in the Correct Direction: Important Clinical Facts Lost in Translation

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Introduction: The immune system recognises cancer cell surface "aberration" via protein, carbohydrate and lipid antigen molecules to induce immune "recognition" of cancer by innate and T-cell receptor mechanisms. However, desired "responsiveness" may be replaced by "tolerance", which facilitates malignant cell growth. We aimed to investigate this paradox.

Methods: Search databases were used to find studies associated with complete clinical responses and survival. Search terms included cancer, effector, regulatory, T-cells, tolerance, responsiveness, inhibition, immune response, survival, complete response.

Results: Numerous studies of solid cancers and systemic therapies reported tolerant/suppressive, or responsive/activated states in patients. Immune therapies, notably Interleukin-2 and CTLA-4 antibodies, did not supply any antigen, but generated durable complete responses, implying endogenous, pre-existing immune responses already occur in the cancer patient before therapy, which when augmented caused successful clinical responses. Partial clinical responses suggest the immune response was forced partially in the correct direction for clinical efficacy, but not efficiently enough. However, of concern, some patients experienced rapid progression of cancer growth, which might be due to unintended tolerance induction from T-regulatory immune stimulation.

Conclusions: Few explanations adequately explain the paradox of the same therapy driving the immune response in either a responsive clinically effective direction, or a tolerant ineffective direction. Susceptibility to immunomodulatory agents appeared to be critically governed by the basic immune reactivity occurring at the time of therapy. Moreover, the detection of "when" the correct time was

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for stimulation of the immune system, therefore appeared absolutely critical for determining the direction the immune response was finally driven and thereby the resultant clinical effect of the treatment. The time when treatment was/is administered is currently not being adequately considered nor determined clinically before therapy is given. This implies that treatment is in essence "random" with respect to its application, despite evidence from the mouse data suggesting an optimum time for dosing actually exists. If we could accurately decide "when" therapy should be applied, many existing and experimental therapies would likely become much more effective clinically. In summary, some near-immediate translational approaches can be readily applied for determination of the correct or optimal timing of therapeutic manipulation of the immune response in the cancer patient for maximum clinical benefit.

Key Words: Immunomodulation, Advanced cancer immune response, Advanced cancer.

Ovarian Tumor-infiltrating T Cells and Myeloid Cells Mediate Immune Suppression Through PD-1/PD-L1 Pathway

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Tumor microenvironment mediates induction of immunosuppressive molecules, such as PD-1 on infiltrating T cells in tumor (TIL), and PD-L1 on tumor cells as well as tumor-derived myeloid cells (TAMs, tolerogenic DC and MDSC). Using a syngeneic mouse model of epithelial ovarian cancer (ID-8 and ID8-VEGF), we assessed the relative contribution of these immunosuppressive molecules in modulating essential TIL function in tumor and ascites. By systematically blocking PD-1 mediated pathways (PD1:PD-L1, PD-1:PD-L2, and PD-L1:B7.1), we found that the level of TIL exhaustion was proportional to the amount of PD-1 ligands expressed by the tumor cells as well as tumor-derived myeloid cells ($r = 0.07525$, $P = 0.0083$). ID-8 ovarian tumor vaccines genetically engineered to express GM-CSF (ID-8-Gvax) or Flt3-ligand (ID-8-Fvax) improved antigen presentation by DC and in combination with PD-1 blockade further increased polyfunctional T cell responses (IFN-γ, TNF-α, IL-2, phospho-T-bet, phospho-Eomes; $P < 0.01$). In addition, in vivo ablation of Treg cells using IL-2DT before tumor inoculation further added value to therapeutic PD-1 blockade ($P < 0.05$). Furthermore, immune activation using anti-4-1BB or CpG-ODN (TLR9 agonist) provided additional antitumor effects. Interestingly, we found an additional role of PD-1 in enhancing Treg cell-mediated suppression in the tumor environment. Hence an effective immune response requires modulation of both suppressive and stimulatory signals.

We thank Drs. James Allison and Michael Curran (GM-CSF and Flt3L plasmids), Dr. Rafi Ahmed and Dr. John Altman (tetramers).

Key Words: Immunosuppression, Ovarian cancer, PD-1.

MIR-124 as a Novel Immunotherapeutic Molecule to Reverse Glioma-mediated Immune Suppression and Enhance Anti-tumor Clearance

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MicroRNAs (miRs) have been shown to modulate critical gene transcripts involved in tumorigenesis, but their role in tumor-mediated immune suppression is unknown. In this study, we evaluated miRNAs that are preferentially down-regulated in malignancy and that interact with immune suppressive pathways as potential new therapeutics. On the basis of miRNA-gene expression of gliomas using tissue microarrays, in situ hybridization, and molecular modeling, we selected miR-124 as the lead candidate for modulating signal transducer and activator of transcription 3 (STAT3), a key molecular hub of tumor-mediated immune suppression. In a glioma tissue microarray, miR-124 expression was significantly down modulated in all grades and types of gliomas relative to normal brain. Upon up regulating miR-124 in glioma cancer stem cells (gCSCs), STAT3 was inhibited; this inhibition reversed tumor-mediated immune suppression, as reflected by an increase in T cell proliferation, Foxp3 + regulatory T cell (Treg) inhibition, and pro-inflammatory immune response up regulation. Treatment of immune-suppressed glioblastoma patient T cells with miR-124 induced a marked effector response. Furthermore, the in vivo local or systemic administration of miR-124 in multiple murine models of glioma, including genetically engineered heterogeneous high-grade gliomas, exerted potent anti-glioma therapeutic effects secondary to STAT3 inhibition in the immune cell population and secondarily enhanced effector responses in the local tumor microenvironment. In summary, miR-124 may be a novel immune-activating agent for glioma treatment (including all grades and types); by exploiting the immune system to mediate direct tumor cytotoxicity, the vexing problem of miR delivery to tumors has been overcome.

Key Words: Immunosuppression, Tumor immunity, Immunotherapy.

Overcoming Tumor-induced Negative Regulatory Pathways in Murine Models of Rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Here, we employ mouse models of embryonal RMS (eRMS) to evaluate the effectiveness of targeting negative regulatory pathways utilized by the tumor to augment immune escape. One of the most critical immune inhibitory checkpoints occurs when Programmed death 1 (PD1) expressed by T-cells binds to its receptor, PDL1. We observe that murine RMS has high surface expression of PDL1 and also induces the expression of PD1 on T-cells in vivo during tumor progression. We find that when we administer anti-PD1 blocking antibodies at the time of tumor inoculation (prevention model) we see 100% long term survival with no sign of tumor formation. When we use a more clinically relevant therapeutic model where anti-PD1 therapy is started at day 7 post tumor inoculation, we observe that much of the beneficial effect of this therapy has diminished. We discovered that RMS tumor-bearing mice have a robust expansion of granulocytic myeloid-derived suppressor cells (gMDSC, CD11b + Ly6G + Ly6Clo) that expressed high levels of the chemokine receptor CXCR2. We hypothesized that the weakened efficacy of anti-PD1 in our therapeutic model may be due to the accumulation of these gMDSC at the tumor site and that CXCR2 mediated their migration. Indeed, we show that when we block this chemokine axis in vitro using anti-CXCR2 or anti-CXCL1/2, we see a significant decrease in ability of gMDSC to migrate toward RMS cell lines. In accordance with this, we find that there are virtually no granulocytic MDSC within the tumors of CXCR2 knock-out mice. In this context, we are able to delay anti-PD1 therapy to day 12 post tumor inoculation and still observe a significant improvement in survival and tumor growth over untreated mice. Importantly, anti-PD1 therapy in wild type mice at this time point proves to be ineffective. Our results demonstrate the efficacy of anti-PD1 immune therapy for eRMS and also the strong influence that tumor-induced MDSCs have against this therapy. Taken together, these data support the notion that multiple negative regulatory

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pathways may need to be overcome before an effective anti-tumor response can be observed.
Key Words: MDSC, PD-1.

Enrichment of CTLA4 + CD39 + CD25 + FOXP3 + Regulatory T Cells in Head and Neck Cancer Patients is Promoted by Therapy With CETUXIMAB and Correlated With Clinical Outcome

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The EGFR-targeted antibody, cetuximab, is clinically effective against head and neck cancer (HNC) in conjunction with chemo/radiotherapy (CRT), but only in 15 - 20% of patients. A better understanding of the influence of cetuximab on the host immune system and the tumor microenvironment, including regulatory T cells (Treg) and NK cell function, may help to increase clinical response rates. Here we report that the frequency of peripheral blood Treg in HNC patients with active disease is significantly increased after cetuximab-based therapy. A significant enrichment of circulating and intratumoral CD4 + CD25 + FOXP3 + Treg were detected, which were increased in tumor-infiltrating lymphocytes (TIL) compared with peripheral blood lymphocytes (PBL). We also observed that immune checkpoint inhibitory receptors (IRs, CTLA-4, TIM-3, PD-1 but not LAG-3) were significantly upregulated on FOXP3 + $CD25^{hi}$ intratumoral Treg when compared to those in peripheral blood lymphocytes (PBL). Moreover, ectonucleotidase CD39 that contributes to generating adenosine, a pivotal immune suppressive metabolite in the tumor microenvironment, was highly upregulated and tightly correlated with phenotype of Treg cells, defined by CD25, FOXP3 and CTLA-4 expression in intratumoral Treg cells. Although patients treated with cetuximab exhibited variable FOXP3, CTLA-4, and CD39 expression on intratumoral Treg cells, their expression levels at baseline were highly upregulated on these suppressive TIL. In addition, cetuximab/NK cells-mediated ADCC was strongly suppressed by autologous CD4 + CD39 + CD25 + Treg cells, which is mediated by Treg-derived TGF-β. Furthermore, lower Treg frequency bfore treatment was associated with better clinical response to cetuximab treatment. Together, our findings reveal that in both tumor and peripheral blood of HNC patients, FOXP3 + Treg cells are highly enriched in the tumor microenvironment by cetuximab treatment and associated with clinical outcome. These results suggest that functional inhibition of T cells using blockade of CTLA-4 or CD39 enzymatic activity may enhance cetuximab immunotherapy by inhibiting immune suppressive activities of Treg cells in the tumor microenvironment.
Key Words: Regulatory T cells, NK cells, Tumor microenvironment.

GM-CSF-induced IL-4Rα Expression on Glioma-infiltrating Monocytes Promotes Immunosuppression and Glioma Growth

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Human epidemiology studies indicate an association of IL-4Rα gene polymorphisms with altered glioma prognosis. We therefore hypothesized that IL-4Rα expression on monocytes plays a significant role in glioma development. We report here that human glioma-infiltrating, but not peripheral CD14 + HLA-DR- cells express high levels of IL-4Rα, suggesting a unique up-regulation of IL-4Rα in the brain tumor microenvironment. Further, IL-4Rα on CD14 + HLA-DR- cells correlates with the expression of immunosuppressive TGFβ and ARG1. We next sought to address the functional significance of IL-4Rα using a murine de novo glioma model. In gliomas induced in wild-type (WT) mice by intracerebroventricular transfection of oncogenes and the Sleeping Beauty transposon, glioma infiltrating CD11b + Gr1 + cells demonstrate increased IL-4Rα compared with peripheral cells. Il4ra-/- mice have prolonged survival compared with WT mice following de novo glioma induction. Consistently, gliomas in WT mice are infiltrated by higher numbers of CD11b + Gr1 + immunosuppressive monocytes than Il4ra-/- mice. Glioma tissues in WT mice demonstrate higher expression of Arg1 and Tgfb than ones in Il4ra-/- mice. Further, anti-Gr1 antibody-mediated depletion of CD11b + Gr1 + cells in WT mice challenged with de novo gliomas led to prolonged survival and tumor regression compared with mice receiving isotype control antibody. We next generated bone marrow (BM) chimeric mice with WT host mice receiving Il4ra-/- or WT mouse-derived BM cells and challenged these mice with glioma cells derived from de novo glioma in WT mice. Consistent with our previous data, WT CD11b + Gr1 + BM cells demonstrated higher degrees of tumor infiltration than Il4ra-/- mouse-derived BM cells, demonstrating that intrinsic CD11b + Gr1 + cell features but not tumor-associated features account for the difference in infiltration into the tumors. We next cultured BM CD11b + cells in the presence of G-CSF and GM-CSF with or without IL-13 to generate CD11b + Gr1 + BM-derived suppressor cells (BMSCs). WT but not Il4ra-/- BMSCs demonstrate increased arginase expression following IL-13 treatment. Consistently, WT but not Il4ra-/- BMSCs can suppress T-cell proliferation in vitro in an arginase dependent manner. Importantly we found that GM-CSF, which up-regulates IL-4Rα expression on cultured BM cells, is indeed up-regulated in both human and mouse glioma tissues. Taken together, in the glioma microenvironment, GM-CSF-inducedIL-4Rα expression on glioma-infiltrating monocytes mediates arginase and Tgf-β production, thereby promoting T-cell inhibition and glioma development.
Key Words: Glioblastoma, GM-CSF, MDSC.

NOS1 Overexpression by Melanoma Cells Contributes to Type I IFNα Signal Dysfunction in Immune Cells

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Dysfunction in type I interferon (IFNs) signaling occurs often in patients with stage II or more advanced cancer and affects responsiveness to IFNα therapy. A marker of such dysfunction is the level of phosphorylation of signal transduction and activator transcription (STAT-1) in peripheral blood mononuclear cells (PBMCs) exposed to IFNα. Such alterations have been recently correlated to predictive and/or prognostic significance. Hypothesizing that this suppression could be partly due to soluble factors released by cancer cells, we screened in a transwell system the effects of a panel of 12 melanoma cell lines on PBMCs obtained from healthy volunteers. After 7 days of co-culture, PBMCs were separated from the melanoma cells and stimulated with IFNα. All but one cell line induced depression of pSTAT-1. Two groups could be identified one inducing stronger suppression (pSTAT-1 low group) than the other one (pSTAT-1 high group). Class comparison between the two groups based on comparative genomic hybridization (CGH) identified a consistent amplification of 12q24 in the pSTAT1 low group. This corresponded to higher transcription of the NOS1 gene included in this genomic region. Administration of NOS donor induced depression of pSTAT-1 levels following IFNα stimulation that was reversed by scavenger

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experiments. NOS inhibitors also reversed the suppression of pSTAT1. This study suggests that NOS1 expression by melanoma cells contributes to type I IFN signal dysfunction in cancer patients and establishes a link between the genetics of individual cancers and a circulating biomarker of potential clinical significance.
Key Words: Immunosuppression, IFNalpha, Melanoma.

Anti-GR-1 Antibody Depletion Fails to Eliminate Hepatic Myeloid Derived Suppressor Cells in Tumor Bearing Mice

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Recent studies show that liver is a preferred organ for the accumulation of myeloid derived suppressor cells. In this study, we examined the effect of systemic RB6-8C5 treatment on hepatic MDSC in tumor bearing mice. EL4-tumor-bearing mice were injected i.p. with RB6-8C5, and hepatic, splenic and blood MDSC were analyzed by flow cytometry. Unexpectedly, hepatic MDSC remained in the liver although RB6-8C5 completely eliminated them in spleen and peripheral blood 24 hours after treatment. Secondary antibody staining confirmed the presence of RB6-8C5-bound MDSC in the liver of mice with subcutaneous tumors. Similar observations were made in two other (colon and melanoma) tumor models. While RB6-8C5 injection induced cell death of hepatic MDSC as shown by AnnexinV/7-AAD staining, these cells were immediately replaced leading to a constant increased frequency of hepatic MDSC. Finally, hepatic MDSC remained immunosuppressive despite RB6-8C5 injection. Our study demonstrates that RB6-8C5 is not suitable for depletion of hepatic MDSC and analysis of their function.
Key Words: Myeloid derived suppressor cell.

Cancer Stem Cells Isolated From Solid Tumors can Display Immunomodulatory Activity for T Cell Responses

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We have previously documented the low immunogenic profile associated with glioblastoma multiforme (GBM)-derived cancer stem cells (CSCs) compared to their FBS-cultured non-CSC (FBS tumor cells) pairs (Di Tomaso et al, 2010). Similar results were obtained for CSCs derived from colorectal cancer (CRC) that we have recently isolated. We could identify two main candidate of negative immunoregulatory pathways associated with CSCs. First, the indoleamine 2,3-dioxygenase (IDO) that was found, by RT-PCR and a colorimetric functional assay, to be up-regulated after IFN-γ treatment preferentially in both GBM- and CRC-derived CSCs versus their FBS tumor cell pairs (7 out of 10 cases). Interestingly, IDO-mediated activity was inhibited by treatment of these cells with the specific inhibitor 1-Methyl Tryptophane (1-MT) or with curcumin. Furthermore, by blocking IDO in GBM CSCs we could both recover T cell proliferation during the co-culture with allogeneic peripheral blood mononuclear cells (PBMCs) from healthy donors and induce TH1 type responses in autologous settings.
Second, we found an over-expression of IL-4 and, though at lower extent, of IL-4R in CRC CSCs compared to autologous FBS tumor cells. We could demonstrate the negative immunoregulatory activity of IL-4 by blocking this cytokine with a neutralizing antibody leading to an efficient in vitro induction of TH1 type responses and recovering of their proliferation both on fresh PBMCs and in the co-culture with autologous CSCs. We are currently exploiting the microRNA signature associated with GBM CSCs to identify microRNA with immunoregulatory functions. Altogether, these results allowed to identify CSC-associated immunomodulatory agents and to demonstrate that their blocking can affect the efficiency of in vitro anti-CSCs T cell responses.
Key Words: Tumor immunity, Indoleamine 2,3-dioxygenase 1, Immunomodulation.

Tumor-derived Adenosine Enhances Generation and Suppressive Functions of Human Adaptive Regulatory T Cells

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Adaptive regulatory T cells (Tr1) are induced in the periphery by environmental stimuli. CD73 expression and adenosine (ADO) production by tumor cells may influence Tr1 generation and their immunosuppressive activity.
Tr1 were generated in co-cultures of CD4 + CD25neg T cells (RC), autologous immature dendritic cells and irradiated ADO-producing CD73 + or non-producing CD73neg breast cancer (BrCa) cell lines (TU). Expression of ectonucleotidases and other surface markers on Tr1 was determined by flow cytometry. Tr1-mediated suppression of RC proliferation was evaluated in CFSE-based assays. Luciferase-based ATP-detection assays and mass spectrometry were used to measure ATP hydrolysis and ADO levels. Cytokine levels were measured by ELISA or Luminex. CD73 expression on tumor cells or T cells in TU tissues was assessed by immunofluorescence.
CD73 + TU induced higher numbers of Tr1 cells than CD73neg TU ($P < 0.01$). Tr1TU73 + hydrolyzed more exogenous ATP, produced more ADO and mediated higher suppression than Tr1TU73neg ($P < 0.05$). ARL67156, an ectonucleotidase inhibitor, and ZM241385, A2A receptor antagonist, reduced suppression of proliferation mediated by Tr1TU73 + cells ($P < 0.01$). Basal-like BrCa cells expressed higher levels of ectonucleotidases and induced more Tr1 than less aggressive luminal-like BrCa.
BrCa producing ADO (CD73 + TU) favor the induction of Tr1 which express CD39 and CD73, hydrolyse ATP to ADO and effectively suppress anti-tumor immunity. In conclusion, ADO emerges as an important novel target to consider in immunotherapeutic approaches in the treatment of BrCa in the future.
Key Words: Cancer immunotherapy, Regulatory T cells, Tumor microenvironment.

Therapeutic Exosome Removal to Target Tumor-mediated Immune Suppression

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Exosomes are 30-100 nm membrane vesicles released by many cells types during normal physiological processes. There is increasing evidence that tumors secrete large quantities of exosomes, which are responsible for the systemic transport of RNAs and immunosuppressive proteins that support tumor growth and metastasis. Using an enzyme-linked lectin-specific assay, we have demonstrated the sensitivity of the lectin Galanthus nivalis agglutinin (GNA), as a binding agent for detection and quantification of tumor-derived exosomes in cell culture media, ascites fluid, and serum. GNA capture is mediated by high mannose glycoproteins abundant on the surfaces of cancer exosomes. This lectin-based capture approach has been applied to a novel device strategy for therapeutic removal of cancer exosomes that is currently in preclinical testing. This device, termed the Hemopurifier, comprises a GNA affinity matrix that is immobilized in the extralumenal capillary space of hollow-fiber plasma filtration membranes in

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plasma separator cartridges that are fitted for existing kidney dialysis systems. Therefore, this device would be applicable for removal of tumor-derived exosomes from the entire circulatory system of cancer patients. Pre-clinical testing using a small-scale Hemopurifier revealed that > 60% of purified ovarian cancer exosomes bound to the GNA matrix during a single pass over the device. Ovarian cancer exosomes applied to cultured Jurkat T cells suppressed the synthesis of activation proteins such as CD3-zeta and JAK-3, as determined in Western blots, thereby demonstrating the immune suppressive activity of exosomes captured with this device. Efficient in vitro capture of exosomes from various types of cancer has been observed in samples from cultured tumor cell lines or plasma from cancer patients that were recirculated over miniaturized Hemopurifier devices. ELISA was used to determine the percentages of exosomes remaining in the samples at defined time intervals of circulation over the Hemopurifier, proving near-complete exosome clearance from samples within 2 hours. Since a spectrum of biologic effects of cancer exosomes have been identified, the Hemopurifier could serve as a method for removing cancer exosomes therapeutically and for defining the clinical impact of exosomes in immunosuppression and tumor growth.

Key Words: Immunosuppression, Tumor immunity, Ovarian cancer.

Invariant Natural Killer T (INKT) Cells Regulate the Response to Radiotherapy and Anti-CTLA-4 by Targeting Dendritic Cells

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Introduction: iNKT cells are powerful immune modulators that have been shown to both activate and suppress adaptive immune response in different settings. In cancer, mechanisms promoting their anti-tumor activity are well characterized but those that dictate their regulatory role remain poorly understood. Accumulated evidence indicate that the interaction between iNKT cells and CD1d + antigen presenting cells, such as dendritic cells (DCs) and macrophages, is a critical determinant of their regulatory function. We have previously shown in a mouse model of poorly immunogenic breast cancer that Jα18-/- iNKT cell-deficient (iNKT-/-) mice had a markedly improved response to treatment with local radiotherapy (RT) and anti-CTLA-4 as compared to wild type (WT) mice. The suppressive function of iNKT cells in WT tumor mice could not be reversed by α-GalCer, a known iNKT agonist and inducer of Th1 cytokines. Here, we studied differences in DC populations between tumor-bearing WT and iNKT-/- mice. We also investigated whether NKT immunoregulation can be reversed by disrupting iNKT activation via CD1d blockade.

Methods: WT and iNKT-/- mice were inoculated subcutaneously with 4T1 tumor cells. On days 13, 19 and 21, mice were euthanized and tumors and draining lymph nodes (dLN) excised for DC analysis. To block CD1d in vivo, WT mice were given 3 doses of anti-CD1d mAb (20H2) on days 3, 7 and 11 post tumor inoculation prior to treatment with IR + anti-CTLA-4, as previously described (Pilones et al, Clin Cancer Res 2009). Mice were followed for tumor growth and survival.

Results: Healthy WT and iNKT-/- mice had similar numbers of DC, but when injected with 4T1 tumor WT mice showed a significant lower number of DC compared to iNKT-/- mice in the tumors ($P = 0.004$) and dLN ($P < 0.05$). Intratumoral DCs from iNKT-/- mice further showed increased expression of maturation markers compared to DC from WT mice. In vitro and in vivo, 20H2 successfully blocked activation of iNKT cells without inducing depletion or reverse signaling of CD1d + DCs. Blockade of CD1d markedly improved the therapeutic response of 4T1 tumor-bearing mice to RT + anti-CTLA-4 resulting in improved tumor regression and survival.

Conclusion: The data suggest that iNKT cells downregulate response to treatment by controlling population of DC present in the tumor and dLN. Since DC are essential for cross-presentation of tumor antigens released by IR-induced cell death, reduced numbers may impair treatment-induced anti-tumor T cell activation. CD1d blockade may offer a novel strategy to release iNKT-mediated suppression and improve response to combination treatment.

Key Words: Immunosuppression, Breast cancer, Immunotherapy.

Indoleamine 2,3-dioxygenase-1 (IDO1) is Expressed in a Subgroup of Childhood Acute Myeloid Leukemias

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Indoleamine 2,3-dioxygenase 1 (IDO1) degrades tryptophan into kynurenine (KYN) and other immune suppressive molecules able to inhibit effector T cells and promote regulatory T-cell (Treg) differentiation and/or activation. We have previously shown that IDO1 is detectable in blast cells from 52% of adult patients with acute myeloid leukemia (AML), in correlation with expanded regulatory T cells (Treg). Furthermore, high copy numbers of IDO mRNA may be a negative independent predicting variable for overall and relapse-free survival in adult AML. We investigated IDO1 expression and function in 16 children with acute leukemia (5 AML, 9 B-cell precursor ALL, 1 infant leukemia with MLL rearrangement and 1 T-cell ALL) and in 1 patient with Ph + chronic myeloid leukemia (CML). Cells from either B-cell precursor or T-cell ALL expressed IDO1 neither constitutively nor after challenge with 100 ng/mL IFN-γ, whereas they up-regulated surface programmed death ligand 1 (PD-L1), an IFN-γ-inducible co-inhibitory receptor. By contrast, leukemia blast cells from 3 out of 5 AML and those from the patient with Ph + CML up-regulated IDO1 protein expression after in vitro challenge with IFN-γ (median 18-fold increase, range 13-95, compared with unstimulated AML cells). The IFN-γ-induced increase of IDO expression was paralleled by STAT3 phosphorylation and was significantly inhibited by pre-treatment of leukemia cells with STAT3 inhibitors (median 1.95-fold compared with unstimulated AML cells, range 0.9-21.5), but not with STAT5 inhibitors. KYN levels significantly increased in supernatants of AML cells stimulated with IFN-γ for 72 hour (18.6 μM/L, range 10.8-26.6) compared with unstimulated cultures (0.9 μM/L, range 0.6-1.3), in parallel with tryptophan consumption (3.2 μM/L, range 0.3-15.3, after challenge with IFN-γ compared with 20.5 μM, range 20.0-37.1, in unstimulated cultures). In a mixed tumor cell lymphocyte culture (MTLC), AML blasts primed with IFN-γ inhibited Th1 cytokine production by allogeneic CD8 + and, to a lesser extent, CD4 + T cells. These effects were potentiated by the addition of exogenous KYN to the MTLC. The provision of D,L-1MT, an IDO inhibitor, to the co-cultures of T cells and AML blasts translated into the restoration of IFN-γ production by both CD4 + and CD8 + T cells.

In conclusion, blast cells from a subset of childhood AML, but not those from B-cell precursor or T-cell ALL, may express functional IDO1 in response to IFN-γ. From a therapeutic standpoint, STAT3 inhibitors may interfere with IDO1 expression in AML cells and break immune tolerance.

Key Words: Tumor immunity, Indoleamine 2,3-dioxygenase 1, Leukemia.

Cyclooxygenase-2 Inhibition Constrains Indoleamine 2,3-dioxygenase-1 Expression by Acute Myeloid Leukemia Cells

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Indoleamine 2,3-dioxygenase 1 (IDO1) is a cytosolic enzyme metabolizing L-tryptophan to kynurenines (KYN), able to induce T-cell suppression either directly or by altering antigen presenting cell function. Cyclooxygenase (COX)-2, the rate-limiting enzyme in the synthesis of prostaglandins, is over-expressed by several tumor types. Both IDO1 and COX-2 affect multiple pathways involved in tumorigenesis, including angiogenesis, invasion, and tumor-induced immune suppression.

We aimed at determining whether COX-2 inhibitors interfere with the IFN-γ-induced expression of IDO1 in leukemia cells. IFN-γ at 100 ng/mL up-regulated COX-2 in HL-60 acute myeloid leukemia (AML) cells, both at mRNA and protein level (average fold-induction of COX-2 mRNA equal to 17.7 ± 1.37 compared with untreated HL-60 cells). The expression of COX-2 mRNA was readily detectable after 24 hours of IFN-γ challenge. The increase of COX-2 protein correlated with heightened secretion of prostaglandin (PG)E2 in culture supernatants. HL-60 cells also up-regulated IDO1 mRNA and protein in response to IFN-γ, and released high amounts of KYN in a time-dependent manner, peaking after 96 hour ($19.21 \pm 9.8\,\mu M$ compared with $1.31 \pm 0.42\,\mu M$ in untreated HL-60 cells). Not unexpectedly, phosphorylated signal transducer and activator of transcription (STAT)-1 was induced in HL-60 cells by IFN-γ treatment, and its expression kinetics and relative amount closely paralleled those of IDO1. STAT3 inhibitors, such as indirubin and STAT3 inhibitor III, but not STAT5 inhibitors or LY294002, a PI3 kinase inhibitor, antagonized the IFN-γ-induced expression of IDO in leukemia cells.

In functional assays, IFN-γ-challenged HL-60 cells promoted the in vitro conversion of allogeneic CD4 + CD25- T cells into bona fide CD4 + CD25 + FoxP3 + regulatory T cells. Pre-treatment of HL-60 cells with 100 μM nimesulide, a preferential COX-2 inhibitor, reduced KYN production in response to IFN-γ by 55% on average ($18.78 \pm 4.02\,\mu M$ compared with $8.75 \pm 3.26\,\mu M$). Exposure to nimesulide also attenuated mRNA signals for IDO1, suggesting that the overall inhibition of IDO activity, leading to suppressed KYN synthesis, might be attributed to the inhibition of IDO1 gene transcription. Finally, nimesulide prevented STAT1 phosphorylation in HL-60 cells, pointing to an involvement of this signaling pathway in the regulation of IDO1 expression.

These data indicate that COX-2 inhibition may constrain the IDO-mediated, immune suppressive tryptophan catabolism and interfere with leukemia-induced immune dysfunction.

Key Words: Indoleamine 2,3-dioxygenase 1, Cancer immunotherapy, Immune escape.

Immunostimulatory Cancer Immunotherapy Regimens Induce Subsequent Potent Immunosuppressive Responses

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We have previously shown that strong immune stimulation using agonistic αCD40 and IL-2 as well as other immunotherapy (IT) regimens result in the induction of robust CD8 + T cell-mediated antitumor responses that are capable of inducing complete tumor regression in various advanced tumor models. We also observed a marked increase in peripheral regulatory T cells with a concordant increase in activation induced cell death of conventional CD4 + T cells during and after therapy. Given this milieu activating and inhibitory signals, we sought to determine the ability of T cells to react to various stimuli during strong immune stimulation such as IT used in cancer treatment. Splenocytes from IT treated mice exhibited significantly blunted proliferative responses to TCR engagement but not cytokine stimulation. CFSE analysis revealed that while CD8 + T cell proliferation and activation marker upregulation were comparable to controls, CD4 T cells failed to proliferate and upregulate CD25. We next investigated primary CD4 responses by mixed lymphocyte reactions (MLR). Mice receiving IT lost the ability to proliferate in primary MLRs compared with controls indicating a profound state of antigen-unresponsiveness. Loss of MLR occurred early during the course of immune stimulation and regardless of combination of αCD40/IL-2 or either treatment singly suggesting that the naïve CD4 + T cell paralysis was a result of strong stimulation. Further analysis of the naïve CD4 + T cell population revealed a concomitant upregulation of SOCS3 in the T cells following IT. SOCS3 is a negative regulator of JAK/STAT signaling, including STAT5 which contributes to CD25 upregulation following TCR mediated activation. Consistent with this, STAT5 phosphorylation was diminished in CD4 T cells restimulated following IT further suggesting a role for SOCS3 in the naïve CD4 paralysis. These data demonstrate that immunostimulatory regimens used in cancer treatment, while inducing potent initial anti-tumor effects, also result in subsequent immunosuppression and immune paralysis affecting primary immune responses.

Key Words: Immunosuppression, Cancer immunotherapy, Naive T cells.

Comprehensive Flow Cytometry Tracking of Lymphocyte Subsets During HD IL-2 Therapy for Melanoma Reveals a Possible Role for ICOS + CD4 + T-regulatory Cells in Limiting Clinical Response

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High dose IL-2 (HD IL-2) has been used as an immunotherapy against metastatic melanoma for over 15 years. However, a lingering question is why it is effective only in a subset of patients and whether predictive biomarkers, before or early during the course of therapy, can be used to improve response rates. In addition, more comprehensive multi-parameter flow cytometry analysis on how lymphocyte and myeloid subsets change during IL-2 therapy is needed. HD IL-2 therapy has been reported to highly expand CD4 + CD25 + Foxp3 + T-regulatory cells (Tregs). However, how Treg cell levels, phenotype, and function change during IL-2 therapy still need further study. We performed a comprehensive multi-parameter flow cytometry analysis of patient blood before and two days after the last bolus of IL-2 infusion during cycle 1 of HD IL-2 therapy. Two lymphocyte subsets expanded the most during the first cycle of therapy: CD4 + CD25 + Foxp3 + Tregs expressing an activation marker, inducible costimulator (ICOS), and CD3-CD56hiCD16loPerforin + NK cells. ICOS + Tregs expressed significantly higher levels of CD25, Foxp3 and had a more activated phenotype than ICOS- Tregs, as indicated by lower levels of CD45RA and CD127. Further phenotypic characterization revealed that ICOS + Tregs had a more suppressive phenotype than ICOS- Tregs, as indicated by higher levels of CD39, CD73, and TGF-β/LAP, and the ability to secrete IL-10, all manifested in a more potent T-cell suppressive function. In addition, almost all ICOS + Tregs were actively proliferating (Ki67 +) after cycle 1 of IL-2 therapy and exhibited an enhanced proliferative response to IL-2 ex vivo relative to ICOS- Tregs. Most ICOS + and ICOS- Tregs expressed Helios, indicating that both Treg subsets are naturally occurring Tregs and not induced Tregs. Further functional analysis revealed that ICOS + Tregs secreted little IFN-γ and IL-2 in comparison to CD4 + Foxp3- cells. Lastly, after analyzing 35 HD IL-2-treated patients at MD Anderson (6 responders and 29 non-responders), we found that non-responders had a significantly higher degree of ICOS + Treg expansion than responders during the first cycle of IL-2 therapy, while there were no significant differences in the ICOS- or bulk Treg population. In

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conclusion, our data underscores the "Treg problem" in HD IL-2 therapy and pinpoint an activated ICOS + Treg subset with a highly suppressive phenotype as the key Treg subset being affected. Our data also suggests that tracking changes in ICOS + Tregs early during the course of HD IL-2 therapy may be a new predictive biomarker.

Key Words: IL-12, Melanoma, Treg cells.

Galectin-1 Knockdown Enhances the Efficacy of Immunotherapy for Murine Malignant Glioma

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There is a growing consensus that the success of immunotherapeutic strategies is limited due to the extensive immunosuppressive environment present at sites of tumors. Insights into the effector molecules that contribute to the establishment of such local immune resistant environment are fundamental in the development of new compounds that sensitize primary tumors to the anti-tumoral effects of immunotherapy. We evaluated the role of tumor-derived galectin-1 in glioma-mediated immune escape and investigated the efficacy of prophylactic immunotherapy in the presence or absence of galectin-1. Galectin-1 is a glycan-binding protein that exerts a plethora of immunosuppressive functions and is overexpressed in several tumors including high-grade glioma.

Methodology: All experiments were performed in the syngeneic GL261 orthotopic glioma model. Stable galectin-1 knockdown was achieved via transduction of GL261 cells with a lentiviral vector encoding a galectin-1-targeting miRNA. Prophylactic immunotherapy was performed with murine bone marrow-derived mature dendritic cells (DC) loaded with total tumor lysate.

Results: Silencing of intratumoral galectin-1 expression prolonged survival of glioma-bearing mice in part by modulating both innate and adaptive antitumoral immune responses. We demonstrated that absence of tumor-derived galectin-1 inhibits the influx of macrophages and myeloid-derived suppressor cells in the tumor micro-environment by modulating CCL2 and VEGF secretion. Moreover prolonged survival required an intact CD4 + and CD8 + T cell response as survival was significantly shortened upon depletion of these cells. Flow-cytometric analysis of the brain-infiltrating immune cell population did not reveal a difference in the total number of CD3 + T cells, however the IFN-γ production was significant increased upon silencing of galectin-1. Finally we demonstrated that tumor- but not host-derived galectin-1 dampens the efficacy of prophylactic DC vaccination.

Conclusion: Collectively these data provide evidence that galectin-1 is an important player in glioma-mediated immune escape by modulating both innate and adaptive antitumoral immunity. Furthermore we demonstrated that local galectin-1 knockdown further boosts the antitumoral immune response induced by immunotherapy. Targeting galectin-1 may offer a novel strategy to sensitize high-grade glioma to the antitumoral effects of immunotherapeutic strategies.

Key Words: Glioblastoma, Immune escape, Active immunotherapy.

Immune Checkpoint Protein Vista as a Novel Target for Cancer Immunotherapy

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V-domain Ig Suppressor of T cell Activation (VISTA) is a newly discovered immune checkpoint ligand that suppresses T cell activation. Our studies demonstrate the suppressive role of VISTA in controlling anti-tumor immunity. In naïve mice and within the hematopoietic compartment, VISTA expression is highly regulated on myeloid antigen-presenting cells (APCs) and T cells. During tumor development, VISTA is highly expressed within the tumor microenvironment (TME), such as on myeloid-derived suppressor cells and Foxp3 + regulatory T cells (Tregs), and directly impairs the generation of optimal anti-tumor immune responses. A specific anti-VISTA monoclonal antibody (mab) neutralized VISTA-induced suppression of T cell responses by VISTA-expressing APCs in vitro. VISTA mab-mediated blockade in vivo suppresses tumor growth by enhancing tumor-specific T cell priming, dampening the activity of Foxp3 + nTregs, reducing the induction of tumor-specific adaptive Tregs, as well as altering the suppressive character of the TME and promoting effector T cell function within the TME. VISTA mab-mediated blockade thus represents a novel and promising strategy for cancer immunotherapy. Studies of VISTA knockout mice are under way.

Key Words: Melanoma immunotherapy, Immunosuppression, Cancer immunotherapy.

THERAPEUTIC MONOCLONAL ANTIBODIES IN CANCER

Activity of Brentuximab Vedotin (Adcetris TM) in Replaced Progressive CD30 + Transformed Mycosis Fungoides (TMF)

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Mycosis fungoides (MF) is the most common subtype of cutaneous T cell lymphomas. Although most patients with MF have a protracted course, some experience a process of large cell transformation called transformed Mycosis fungoides (tMF) which in some cases express CD30. This is often associated with an aggressive course requiring more aggressive and sometimes intensive therapies. Given the rarity of the disease, clinical studies to compare the efficacy of available treatment options are lacking and the majority of such cases end up receiving therapies geared to peripheral T-cell lymphomas.

More effective therapies are needed based on the molecular pathogenesis of the disease. Brentuximab Vedotin (AdcetrisTM), which is a anti-CD30 antibody drug conjugate, is approved for the treatment of relapsed and/or refractory systemic anaplastic large cell lymphoma and Hodgkin's disease, where the expression of CD30, its target, is expressed.

We investigated the activity of Brentuximab Vedotin in a case of relapsed and rapidly progressive CD30 + tMF. Our patient was diagnosed with tMF in 1999 and has been controlled with radiotherapy, oral Bexarotene, topical Nitrogen mustard sequentially until 8 months prior to presentation when the disease underwent rapid progression and biopsy reconfirmed the transformation to CD30 + large cell variant. Once again his MF lesions responded well to topical Bexarotene, Clobetasol and NBUVB.. However, his tMF nodules progressed rapidly. NBUVB was stopped and he was started on Brentuximab at a standard dose of 1.8 mg/kg IV every 21 days. After treatment with 3 cycles, there was a complete clinical resolution of both his tMF and classical MF skin lesions. Currently the patient continues with his 5th cycle of Brentuximab which he has tolerated so far with mild transient post-infusion fatigue as well as mild cytopenias and mild intermittent peripheral sensory neuropathy, expected side-effects of the treatment. More importantly his disease continues to be in complete clinical remission as of his last visit.

Brentuximab seems to have significant activity on its own in tMF that expresses its target, CD30. Its incorporation into front line treatment of patients with this disease needs further study and validation.

Key Words: Antibody response, Lymphoma, Targeted therapeutics.

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Development of a New ADCC-like Assay and its Clinical Values of the Prediction of Trastuzumab Responses

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Trastuzumab is a monoclonal antibody drug against HER-2, and has been widely used to treat HER2-positive breast cancers. However, certain population of HER-2 positive patients fail to respond to trastuzumab. Antibody-dependent cell-mediated cytotoxicity (ADCC) has been shown to be one of the modes of action for trastuzumab. Thus, the purpose of this study was to investigate the inter-individual differences in trastuzumab-mediated ADCC activity, and develop a new assay to quantitative ADCC to predict the efficacy of trastuzumab, because traditional ADCC is not applicable to routine diagnostic test.

Using the peripheral blood mononuclear cells (PBMCs) of three healthy volunteers (HVs), we first examined ADCC. One of the HVs showed the highest ADCC against the HER2 positive BT-474 and MCF-7 cells, and we found in another independent experiment that the inter-individual differences among three subjects is consistent. These inter-individual differences of ADCC were also confirmed using PBMCs of an additional 8 HVs in three independent experiments. To search the biomarkers which correlate with ADCC activity, we adopted an new ex vivo gene expression assay. We examined the expression change of 14 candidate leucocyte genes in the 8 HVs after ex vivo exposure to heat-aggregated IgG1 for 4 hours. We found that the values of fold increase (FI) in expressions of TNFSF15, IL-6, and CxCL3 are significantly correlated with ADCC activity ($R = 0.74$, $R = 0.85$, $R = 0.87$, respectively). Next, we evaluated prospectively whether FIs of these 14 genes are associated with a pathological complete response (pCR) in 18 patients who received trastuzumab-based neoadjuvant chemotherapy. Patients who achieved pCR had higher FI of CXCL-1, CXCL-3, TNFSF-2, and TNFSF-15 than those who did not ($P = 0.004$, 0.015, $= 0.0495$, and $= 0.014$, respectively). This is the first report of the consistent analysis of inter-individual differences in trastuzumab-mediated ADCC activity in vitro, and of a promising new assay for predicting the ADCC activity as well as the pathological response to trastuzumab-based neoadjuvant chemotherapy.

Key Words: ADCC.

Activity of Rituximab-bendamustine (RB) in Sequence With Brentuximab Vedotin (Adcetris TM, BV) in Gray Zone Lymphoma (GZL) Between Hodgkins Lymphoma (HL) and Diffuse Large B Cell Lymphoma (DLBCL)

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GZL are rare subtypes of lymphoma characterized by overlapping morphological and immunophenotypical features of HL and Non-Hodgkins Lymphomas (NHL). GZL between HL and DLBCL is the most frequently encountered. While Rituximab (anti-CD20 Antibody) Bendamustine combination has been shown to have significant activity in a wide range of B-cell lymphomas and Bv (anti-CD30 antibody drug conjugate) in relapsed/recurrent HL, there is no standard or consensus in the literature regarding GZL therapy. It has been a common practice that such cases get treated with DLBCL combinations.

We present a unique case of GZL between HL and DLBCL that was treated successfully with sequential RB followed by Bv. Our patient is 62 year old male who was initially diagnosed with DLBCL. The patient then was treated with R-CHOP with complete remission though he allegedly had a hard time tolerating this regimen. After five years he was found to have retroperitoneal, mesenteric, pelvic and inguinal lymphadenopathy. Excisional biopsy revealed lymphoproliferative disorder consistent with GZL between HL and DLBCL with notable CD20 and variable CD30 positivity. The patient refused to take R-CHOP and agreed to try RB instead. He was treated with 6 cycles of RB. Re-staging PET/CT scans revealed near complete resolution with some residual disease, which we presumed was due to the CD30 + component. The patient agreed to 3 cycles of Bv to address his CD30 + disease which lead to achieving complete PET/CT remission and prompting a transplant referral. The patient underwent adequate stem cell collection and subsequently successful engraftment. This is the first report in the literature documenting the activity of RB followed by Bv in GZL between HL and DLBCL which was well tolerated with mild expected side-effects of both regimens leading to a successful transplant. More clinical trials are warranted to confirm and validate the activity of such regimen in this rare lymphoma subtype.

Key Words: Antibody response, Lymphoma, Targeted therapeutics.

Epidermal Growth Factor Receptor Signaling Facilitates Immune Escape Function in Head and Neck Cancer

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Head and neck cancer (HNC) cells express low HLA class I and antigen processing machinery (APM) components, which is a major immune escape strategy from T cell lysis. However the mechanism of this immune escape strategy by HNC is largely unknown. Epidermal growth factor receptor (EGFR) is the most validated tumor antigen (TA) target for HNC. We show that FDA approved EGFR blocking mAb cetuximab enhanced expression of HLA class I and APM components in tumor cells, which was associated with the EGFR expression level on HNC cells. Interestingly, EGFR signaling blocking with cetuximab also enhanced IFN-gamma receptor on SCCHN cells and augmented induction of HLA class I by IFN-gamma. Upregulation of HLA-B and C allele was more pronounced than HLA-A allele after cetuximab treatment. Moreover, EGFR signaling blockade enhanced the level of TAP-1/2 in a STAT-1 $^{+/+}$ cell line but not in STAT-1 $^{-/-}$ cell line documenting the STAT-1 dependence of cetuximab's effect. In addition, cetuximab treatment enhanced the recognition of tumor cells by $EGFR_{853\text{-}861}$-specific CTL and also enhanced surface presentation of non-EGFR TA such as $MAGE\text{-}3_{3271\text{-}279}$. These findings describe a novel immune escape function associated with EGFR signaling and the reversal with cetuximab, which may help to better optimize the selection and clinical outcomes for on-going mAb-based immunotherapy.

Key Words: Tumor immunity, Immune escape, Tumor associated antigen.

A Study of Immune Mechanisms of Action of Anti-epidermal Growth Factor Receptor Antibodies Cetuximab and Panitumumab and its Implication in Head and Neck Cancer Therapy

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Epidermal growth factor receptor (EGFR) targeted therapies are modestly effective in the treatment of head and neck cancer (HNC). The anti-EGFR monoclonal antibodies cetuximab (IgG1) and panitumumab (IgG2) block EGFR signaling through a common ligand binding epitope, yet to date only cetuximab has shown an

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increase in patient overall survival in HNC clinical trials, suggesting differences in mechanisms of action. We show that both cetuximab and panitumumab trigger similar anti-proliferative effects on various HNC cells in vitro, induce EGFR-specific HLA-ABC expression with similar efficacy ($P < 0.05$) and generate same anti-idiotype Ab in treated patients. However, only cetuximab treated HNC cells enhance the upregulation of pro-inflammatory cytokines in PBMC, and induce NK cell activation markers such as IFN-gamma CD137, CD69, CD107a, and ICAM-1 ($P < 0.05$), whereas panitumumab fails to activate NK cells through CD16 activation. Indeed, panitumumab abrogates cetuximab induced ADCC ($P < 0.05$), but panitumumab shows modest cytotoxic activity through myeloid cells alone, but not NK cell. Moreover, co-culture of DC with cetuximab-activated NK cell enhances the expression of HLA-DR, CD86, CD83 maturation markers on DC ($P < 0.05$) and stimulated secretion of IL-12p40/70 and IFN-gamma($P < 0.05$). Furthermore, cetuximab-induced NK: DC cross-talk enhanced frequency of cytotoxic T lymphocytes (CTL) in vitro, and was associated with higher frequency of EGFR-specific CTL in treated HNC patients ($P < 0.05$). These contrasting immune mediated events between between cetuximab and panitumumab may help to explain the differential clinical activity of these mAb therapies, provide biomarkers of clinical response, and inform potential strategies to improve their efficacy and clinical application.

Key Words: Cancer immunotherapy, ADCC, EGFR inhibitors.

Targeting Regulatory T Cells by Intracranial Convection-Enhanced Delivery of Anti-CD25 Promotes Tumor Clearance in Murine Glioma

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Introduction: A hallmark of glioblastoma (GBM) is subversion of the cellular immune response, a process partially mediated by tumor-infiltrating regulatory T cells (Tregs). Though Tregs are susceptible to therapeutic targeting by anti-CD25 antibodies, the limited permeability of the blood-brain barrier to antibodies presents a challenge for this approach. We hypothesized that intracranial convection-enhanced delivery (CED) of anti-CD25 monoclonal antibody would improve local antibody delivery and augment the efficacy of this immunotherapeutic strategy in an animal model of GBM.

Methods: Mice underwent intracranial injection of a PDGF-expressing retrovirus and were treated on day 14 following tumor induction with single-bolus or continuous delivery (via osmotic minipump) of anti-CD25 antibody, delivered either systemically (by intraperitoneal injection) or intracranially. Subsets of animals were sacrificed at pre-determined time points for analysis of intratumoral T cell infiltrates and peripheral cellular immune function. Survival was evaluated in additional cohorts of animals to compare the relative clinical benefit of each treatment strategy.

Results: We found that CED of anti-CD25 directed intratumoral lymphocyte populations toward a pattern canonically associated with immune tumor clearance. Intratumoral Tregs were significantly lower after two weeks of treatment ($0.11 \pm 0.08\%$ of CD4 + T cells) when compared to controls ($33.17 \pm 1.71\%$ of CD4 + T cells; $P < 0.0001$) and intratumoral CD4:CD8 ratios in treated mice were more strongly biased toward an effector T cell phenotype (0.43 ± 0.042 in treatment group vs. 0.77 ± 0.074 in controls; $P = 0.005$). Additionally, CED immunotherapy slowed tumor progression and conferred a significant survival benefit over equivalent-dose administration of anti-CD25 mAb by intracranial single-bolus injection (43.5 d, $P = 0.0016$), systemic single-bolus injection (66 d, $P < 0.0001$) or systemic continuous delivery (56 d, $P < 0.0001$). Interestingly, we noted attenuation of peripheral Treg expansion and associated improvement for in vitro cellular immune function in all treatment groups relative to controls, an effect that was particularly robust in animals undergoing intracranial CED of anti-CD25.

Conclusions: These results demonstrate that sustained intratumoral delivery of anti-CD25 can improve the efficacy of immunotherapy for malignant gliomas. Convection-enhanced delivery may allow for significant optimization of this approach.

Key Words: Glioblastoma, Treg cells.

TUMOR MICROENVIRONMENT

Novel Dual Mode Fluorine MRI, NIR Fluorescent Probe for Non-invasive Detection of Tumor-associated Inflammation

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Tumor associated macrophage are active in both tumor progression and remission processes, affecting prognosis depending on the nature of their involvement. Imaging macrophage recruitment and persistence to gauge tumor from normal host tissue is an area of great interest. Herein we propose the use of a novel dual mode fluorine magnetic resonance imaging (MRI), near infrared (NIR) fluorescent probe for the non-invasive detection of tumor associated inflammation. The use of this technique allows for rapid optical assessment of tumor associated macrophage as well as the specificity and detailed resolution provided by pairing 19F and conventional 1H MRI. Upon administration of the 19F/NIR reagent, tumors were visible at 6 hours. Both the liver and spleen were also visible due to clearance through the reticuloendothelial system. Use of the dual mode reagent was also compatible with MRI visualization, where detection of inflammation was in the periphery of, and not integral to, the tumor itself, information that could not be detected by optical means. Upon resection of the tumor, liver, spleen and other regions of interest fluorescence detection within the organs/tissue correlated with fluorine content and agreed with macrophage infiltrates and regions of reagent clearance.

Flow cytometric analysis of whole blood show the preferential labeling of the macrophage population and immunofluorescent analysis of labeled macrophage further confirms cellular labeling. Dual functioning contrast agents enable both quick monitoring and sensitive quantification when evaluating the tumor microenvironment and potential changes in macrophage infiltrates as a result of therapeutic intervention.

Key Words: Cell trafficking, Macrophages.

Intratumoral Delivery of Interleukin-12 DNA With in vivo Electroporation can Lead to Regression of Injected and Non-injected Tumors in Merkel Cell Carcinoma: Results of a Phase 2 Study

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Background: Local delivery of immunostimulatory cytokines to the tumor microenvironment (TME) may spare systemic toxicity and may improve efficacy due to adequate cytokine concentration in the vicinity of tumor antigens. Interleukin-12 (IL-12), a master regulator of adaptive type-1 cell-mediated immunity, is associated with promising antitumor efficacy, but its utility is restricted due to serious adverse events (AEs) associated with systemic administration. Promising results were noted in a phase 1 trial of intratumoral (IT) injection of IL-12 plasmid DNA (pIL-12) followed by in vivo electroporation (EP) in patients (pts) with melanoma {Daud AI. J Clin Oncol. 2008}. We report the preliminary results of a phase 2 multicenter trial of pIL-12 EP in pts with Merkel cell carcinoma (MCC), an aggressive virus-associated malignancy.

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Methods: 15 MCC pts with a superficial injectable tumor will be enrolled to receive pIL-12 EP treatment delivered on days 1, 5 and 8 of each cycle. Tumor biopsies and peripheral blood (PB) samples will be collected in all pts at baseline and post-treatment. Pts with localized MCC may receive 1 cycle of pIL-12 EP followed by definitive surgery and/or radiation therapy (RT) starting during weeks 3-4 (Arm A); pts with distant metastatic disease may receive multiple cycles every 6 weeks (Arm B). Primary endpoint is post-treatment change in IL-12 protein level in the TME. Secondary endpoints include safety, clinical efficacy (including objective responses in injected and distant lesions), and cellular and humoral immunologic changes in the TME and PB.
Results: 5 pts have been enrolled to date to Arm B. Four pts have completed one (n = 2) or two (n = 2) cycles. Treatment has been tolerated well. Treatment-related AEs include transient grade 1 pain (n = 5) and grade 1 injection site reaction (n = 1) without any systemic or residual toxicity. Three patients had progressive disease as the best response. One patient with baseline progressive MCC despite multiple prior therapies (systemic chemotherapy, surgery, RT, IT interferon) has had a confirmed partial response (> 70% regression) that is ongoing at 6 + months. The regression of injected as well as non-injected tumors, along with no new tumors over 6 months, suggest successful induction of systemic immune response from local IT immunotherapy in this patient. Updated clinical and correlative results will be presented at the meeting.
Conclusion: Preliminary results indicate that IT immunotherapy with pIL-12 EP in MCC patients is tolerated well and may lead to induction of systemic antitumor immune responses.
Key Words: Merkel cell carcinoma, Cytokine, Phase II.

Function and Prognostic Role of Indoleamine 2,3-dioxygenase in Endometrial Carcinoma

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Indoleamine-2,3-dioxygenase (IDO) catalyzes the degradation of the essential amino-acid tryptophan. In this way, IDO suppresses the function of T-lymphocytes and is an important immune escape mechanism for cancer. It is to be expected that IDO influences prognosis of cancer patients. IDO-induced suppression of T-lymphocytes can be reversed by the pharmacological inhibitor of IDO, 1-methyltryptophan (1-MT). Possibly, anti-IDO agents are an interesting new approach in order to optimize treatment and prognosis of, for example, endometrial carcinoma (EC) patients. Over the last years, not much progress has been made in improving treatment and prognosis of EC patients and therefore, new therapeutic strategies are needed.
This study investigated the suppressive mechanism of IDO and the prognostic influence of IDO expression in EC patients using immunohistochemistry, electron microscopy and XLC-MS/MS on tumor cell lines and a tissue microarray containing primary EC tissue of 355 patients. Previously determined numbers of intratumoral CD8 + and Foxp3 + T-lymphocytes were correlated to IDO expression.
We showed that IDOhigh expression was present in 18.4% of primary EC tissue. IDOhigh expression was associated with lower numbers of intratumoral CD8 + T-lymphocytes ($P = 0.031$). Electron microscopy showed that IDO was exclusively localized below the cellular membrane. Extracellular concentrations of tryptophan decreased dramatically (factor 339) after treatment with IFN-gamma while kynurenine concentrations increased (factor 25). Intracellular changes were less significantly (factor 2). Next to well-known prognostic parameters, IDOhigh expression was independently associated with poor disease specific survival in the general cohort of EC patients (HR 2.62, 95% C.I. 1.48-4.66, $P = 0.001$) and among patients with early stage EC (HR 3.06, 95% C.I. 1.10-8.54, $P = 0.032$).
In summary, we investigated the role of IDO expression in a EC patients. We showed that IDOhigh expression was associated with decreased numbers of CD8 + T-lymphocytes and a decreased DSS. Our results might help to understand why T-lymphocytes are more prone for apoptosis compared to cancer cells. New cancer treatment strategies are currently under investigation, including the use of IDO-blocking agents.
Key Words: Indoleamine 2,3-dioxygenase 1, Immune escape, Tumor infiltration lymphocytes.

Vaccination into the Tumor Microenvironment Using Recombinant Vaccinia Expressing HER2/NEU Leads to Tumor Regression and the Generation of a Tumor-specific Systemic T Cell Response in a Mouse Model of HER2/NEU-overexpressing Mammary Carcinoma

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The goal of cancer vaccination is to develop a tumor-specific immune response capable of overcoming immune escape mechanisms that induce systemic anergy. Our studies have demonstrated an overlapping repertoire of immune escape mechanisms in models of murine bladder (MB49) and breast tumors. Orthotopic growth of a syngeneic HER2/neu-overexpressing mammary carcinoma in FVB mice developed in our laboratory (NBT1) is associated with an increase in Gr1 + CD11b + myeloid derived suppressor cells (MDSCs) in both the tumor microenvironment and systemically. VVneu and VVGMCSF are recombinant vaccinia viruses produced in our laboratory which encode HER2/neu and GM-CSF, respectively. In naïve FVB mice, vaccination subcutaneously or into the mammary fatpad with the combination of VVneu and VVGMCSF resulted in a similar increase in the HER2/neu-specific systemic and local CTL response, as measured by cytolytic activity of restimulated systemic (splenic) and vaccination-site draining lymph node (VDN) effectors and HER2/neu-specific MHC Class I tetramer flow cytometry. Orthotopic growth of NBT1 in untreated FVB mice failed to induce a systemic anti-tumor CTL response. When VVneu and VVGMCSF were injected, along with Keyhole Limpet Hemocyanin (KLH), directly into the tumor or subcutaneously in the contralateral side of NBT1-bearing mice, only vaccination into the tumor microenvironment resulted in a statistically significant increase in CTL activity and regression of NBT1. Moreover, the percentage of systemic MDSCs decreased in mice that received vaccination into the tumor microenvironment, while remaining stable in mice treated with contralateral subcutaneous vaccine. These results demonstrate a dual role of the tumor microenvironment in both promoting systemic anergy against HER2/neu and providing an effective vaccination site that allows a reversal of tumor-specific anergy. The studies also demonstrate that immunization with antigen-encoding recombinant poxvirus vaccines into the tumor microenvironment, but not systemically, can be effective in reversing MDSC-associated anergy.
Key Words: Cancer vaccine, Myeloid derived suppressor cell, Tumor microenvironment.

Immunophenotypic Analysis of Tumor Infiltrating T Lymphocytes and Modulation of Antitumor Immunity in Patients With Breast Cancer: Correlation With Clinicopathological Features

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Breast cancer is the most common form of cancer in females. It is estimated that the disease will affect five million cases worldwide over the next decade. Leukocyte infiltration into tumors is considered one of the hallmarks of cancer development. The presence of tumor-specific CD4 + Treg cells at tumor sites may play a significant role in the suppression of antitumor immunity. However, interferon- γ (IFN-γ) released by tumor infiltrating lymphocytes (TILs) is involved in effective anti-tumor immune responses mediated by modulating both adaptive and innate immunity.

In this study, we used flow cytometry to determine the phenotype and relative abundance of the TILs in tumor specimens from breast cancer patients. The expression of both effector CD4 and regulatory markers on the TILs were determined using monoclonal antibodies. The anti-tumor response was evaluated by measuring IFN-γ levels in culture supernatants of the freshly isolated TILs. We correlated the percentages of TILs and their culture supernatant levels of IFN-γ with various clinicopathological parameters of the patients.

The immunophenotypic analysis of the isolated TILs obtained from breast tumor tissue specimens showed different types of cell populations; identified by markers of differentiation; CD4 + and CD4 + /CD25 + cell sub-populations which represented 11.7 ± 10.9% and 2.8 ± 4.6% respectively. The mean CD4% was significantly higher ($P = 0.013$) in patients with PR negative in comparison with PR positive. It was also higher in patients with negative lymph node metastasis than positive lymph node metastasis but this was not statistically significant. In addition, we found that there was highly significant increase in culture supernatant IFN-γ levels in postmenopausal patients than premenopausal patients ($P = 0.013$), in early stages (I + II) than late stages ($P = 0.012$) and in tumors with negative vascular invasion ($P = 0.003$). There is an association between high percentages of tumor infiltrating T regs and larger tumor sizes. A significant positive correlation was found between IFN-γ levels in TIL culture supernatants and CD4 + % ($P = 0.007$). While, there was no correlation between IFN-γ levels in TIL culture supernatant and CD4 + CD25 + T regs %. These findings shoud help in the design of clinical trials that manipulate the tumor microenvironment to the advantage of the host. Regarding the critical role of IFN-γ as a key director of immune response in breast cancer, it could be considered as a potential therapeutic tool in the disease.

Key Words: Breast cancer.

Cysteamine Inhibits Invasion, Metastasis and Extends Survival by Down-regulating Matrix Metalloproteinases *in vivo* Mouse Model of Human Pancreatic Cancer

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Cysteamine is an anti-oxidant aminothiol and a choice drug for the treatment of nephropathic cystinosis, an unusual lysosomal storage disease. Cysteamine is a chemosensitization and radioprotection agent and its antitumor effects have been investigated in various tumor cell lines *in vitro* and chemical carcinogenesis models *in vivo*. In the present study, we have examined if cysteamine has anti-tumor and anti-metastatic effects in transplantable human pancreatic cancer, an aggressive metastatic disease. By matrigel invasion assay, we studied anti-invasion effects of cysteamine and cell migration in ten pancreatic cancer cell lines. To study mechanism of action, we examined its effect on cell viability and matrix metalloproteinases (MMPs) activity. The anti-metastasis effect was examined in two orthotopic mouse models of human pancreatic cancer by measuring peritoneal metastasis and survival of animals. Cysteamine suppressed both migration and invasion of all ten pancreatic cancer cell lines at concentrations (< 25 mM) that caused no toxicity to cells. It also caused a significant decrease in MMPs activity (IC50 38 - 460 μM) and xymographic gelatinase activity in a dose dependent manner *in vitro* and *in vivo*. In two established pancreatic tumor models in mice, cysteamine significantly decreased metastasis, although it did not affect the size of primary tumors. Furthermore, cysteamine prolonged survival of mice in a dose-dependent manner without causing any vital organ toxicity. We also observed that MMP activity was significantly decreased in animal tumors treated with cysteamine.

Cysteamine had no clinical or laboratory adverse effects in the host even at the highest dose. Based on these findings, we believe that cysteamine, an agent with a proven safety profile, may be useful for inhibition of metastasis and prolonging the survival of a host with pancreatic cancer.

Key Words: Metastases, Animal model, Tumor microenvironment.

Natural History of Tumor Growth and Metastasis in Common Spontaneous Murine Breast Cancer Models

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Background: MMTV, C3(1)-Tag, and DMBA are three commonly used mouse models, representing HER2/neu + , HER2-/ER-/PR-, and HER2-/ER + /PR + breast cancers, respectively. The wide use of these models prompted our study of the natural progression and incidence of tumor growth, frequency and localization of metastases, and characterization of the infiltrating T-cells in the tumor microenvironment in each of these models.

Methods: MMTV and C3(1)-Tag mice (n = 64 and 52, respectively) were observed for naturally occurring breast tumors, and DMBA mice (n = 48) were treated with a carcinogen at 8 weeks of age and observed for chemically induced breast tumors. All tumors were measured three times per week until sacrifice. Incidence of metastasis was measured in H&E sections prepared from femur, liver, brain and lung samples. Tumor infiltrating CD4 + , CD8 + , T regulatory Foxp3 + , and myeloid derived suppressor cells (MDSC) were measured by flow cytometry.

Results: Age of tumor onset, tumor incidence, and kinetics of tumor growth were significantly different ($P < 0.05$) between the three mouse models. Significantly different (slow, intermediate, and fast) tumor growth rates within each model were also observed (Mann-Whitney Test). Lung was the predominant metastatic site in all models (28.6%, 12.9%, and 40% incidence in MMTV [n = 8/28], C3(1)-Tag [n = 4/31], and DMBA [n = 6/15] models, respectively). In the DMBA model, metastases were also detected in liver (6.7%) and femur (7.7%). Tumor-infiltrating CD4 + , CD8 + , Foxp3 + , and MDSC percentages were also significantly different between models (unpaired *T*-test).

Conclusion: Our observation of significantly different tumor growth rates in all models indicates biologically relevant tumor heterogeneity. Ongoing analysis aims at correlating tumor infiltrates with tumor growth rates. More aggressive metastasis was observed in the carcinogenic DMBA model as compared to the spontaneous tumor models.

Key Words: Breast cancer, Metastases, Animal model.

The Effect of Tumor-derived Arginase on T Cell Suppression and Tumor Progression

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While many cancer patients have sustained cancer remissions, others experience a rapid and fatal progression. What makes tumors quiescent as opposed to aggressively tumorigenic or metastatic is a critical question that most likely has several answers. It is

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known that immune cells have the ability to destroy cancer cells and that infiltration of T cells in the tumor mass is the most significant predictor of survival. However, in order for T cells to kill tumor cells, they must become activated against tumor antigens. Tumors grow in an intricate microenvironment that is immune suppressive. One of the contributing factors to anti-tumor T cell tolerance is the production of arginase by tumor-associated myeloid derived suppressor cells. Arginase activity has also been detected in human tumors and tumor cell lines, but the role of tumor cell derived arginase as a factor that promotes tumor progression is not well understood. Using the FVB MMTV/Neu murine model of breast cancer, our laboratory cloned primary epithelial and mesenchymal cell lines from spontaneous mammary tumors. While there was no difference in arginase activity between primary epithelial and mesenchymal cells, there was a 25-fold increase in arginase I transcript and a 16-fold increase in arginase I activity in an established epithelial tumor cell line as compared to the primary cell lines. Importantly, when administered as a cell-based tumor vaccine, the established cell line with high arginase activity was associated with reduced IFN-γ production by splenic tumor-specific CD8 T cells as compared to the primary epithelial and mesenchymal cell lines. When orthotopically inoculated with 50,000 tumor cells, mice ($N = 4$) that received the cell line with high arginase activity succumbed to tumor growth significantly faster than mice that received a primary epithelial cell line ($P < 0.01$). Given these data, we hypothesize that tumor-derived arginase activity impairs T cell function and is a factor that contributes to tumor progression. To test this hypothesis, the primary epithelial cell lines have been permanently transfected to over-express arginase I, and the established epithelial tumor cell line has been transduced with an arginase I miRNA lentiviral vector to knock-down arginase I activity. These cell lines as well as manipulation of arginase activity using nor-NOHA as an arginase inhibitor, will be used to further dissect the role of tumor-derived arginase on T cell function and tumor progression. It is conceivable that arginase activity in tumor cells may be an important target for therapeutic intervention, and may be a critical biomarker used to predict which tumors will rapidly progress versus those that will remain dormant.
Key Words: Immunosuppression, Breast cancer, Tumor microenvironment.

Characterization of Intra-tumoral Immunity in Common Cancers: CD146 Expression in CD4 T Lymphocytes

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Tumors develop immune escape mechanisms promoting survival and growth which represents a major obstacle to the success of immunotherapy. We have tried to better understand the intra-tumor immunological environment to elucidate immune tolerance mechanisms. We first established an optimal tumor tissue disaggregation method for human infiltrating immune cell (TIIC) characterization. We then adapted this method to characterizing the phenotype and functions of cells expressing the CD146 adhesion molecule. CD146 + cells are known to secrete various immuno-modulatory cytokines in pathologies like multiple sclerosis and rheumatoid arthritis. However, the role of these cells remains to be described in cancer. We initially compared the effects of three tissue disaggregation methods on TIIC biology by evaluating cell death, loss of cell surface markers, and inhibition of cell proliferation. Then, using samples from breast, kidney and lung cancer patients, we performed phenotypic characterizations of immune cells expressing CD146 by flow cytometry by comparing tumors to peripheral blood. Mechanical disaggregation by Medimachine™ appears more efficient in preparing TIIC with minimal phenotypic alterations. Furthermore, immune cells expressing CD146 represent a small but non-negligible fraction of cells in the blood. This population is enriched in TIICs. CD146 + cells appear to be mostly CD4 T lymphocytes of which many possess a regulatory CD25 + FOXP3 + profile. The study of regulatory immune populations expressing CD146 is of interest to develop neutralization strategies which may lead to the improvement of tumor immunotherapies
Key Words: Tumor infiltration lymphocytes.

Delicate Balance Among Three Types of T Cells in Concurrent Regulation of Tumor Immunity

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Many studies have demonstrated the importance of regulatory cells such as Tregs and type II NKT cells in the immune regulation of cancer. However, it is still not clear why different suppressive cells play a dominant role in different tumor models. Here, we examined the relative role of the two suppressors, Tregs and type II NKT cells, in a subcutaneous CT26 tumor model in three strains of mice: wild-type, NKT cell-deficient CD1dKO mice and Jα18KO mice, which lack type I NKT cells but still retain type II NKT cells. Tumors grew in all three strains. Treg blockade led to tumor rejection in WT and CD1dKO, but surprisingly not in Jα18KO mice, suggesting that Tregs are not necessary for the suppression of tumor immunity in Jα18KO mice. Based on our previous findings that type I and type II NKT cells can counter-balance each other, we hypothesized that in WT mice type I NKT cells neutralize the effect of type II NKT cells, leaving Treg cells as the primary suppressor, whereas in Jα18KO mice, unopposed type II NKT cells suppress tumor immunity even when Tregs are blocked. We confirmed this by blocking both suppressors, Tregs and type II NKT cells using anti-CD25 and anti-CD1d, as well as by reconstituting type I NKT cells in Tregs-depleted Jα18KO mice. Moreover, shifting the balance between the two types of NKT cells by stimulating type II NKT cells with sulfatide in WT mice abrogated the protective effect of Treg blockade. We conclude that in the absence of type I NKT cells, blockade of both type II NKT cells and Tregs is necessary to abrogate the suppression of tumor immunity, and that a third cell therefore determines the relative roles of these two regulatory cells in a delicate balance. As cancer patients often have defects in type I NKT cell functions, controlling both suppressors may be critical for the success of immunotherapy of human cancer.
Key Words: Immunosuppression, Tumor immunity, Regulatory T cells.

Inhibitory Receptors on Tumor Infiltrating Lymphocytes Reflect Aberrant TCR Triggering in the Tumor Microenvironment

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A family of T cell inhibitory receptors limits T cell functions by negatively regulating signals mediated by T cell antigen receptor (TCR) and contributes to T cell dysfunction in tumor micro-environment. Despite emerging appreciation for their important roles in regulating the effector functions of tumor-infiltrating lymphocytes (TIL), the underlying mechanisms for regulating inhibitory receptors expressed on TIL remains to be fully elucidated. We herein examined the expression pattern of CTLA-4, PD-1, TIM-3, and LAG-3 on TIL and compared to that of peripheral blood T lymphocytes (PBL) in patients with head and neck cancer (HNC) caused by either carcinogen exposure or by human papillomavirus (HPV). Here, we report that both $CD4^+$ and $CD8^+$ TIL predominantly express PD-1 and TIM-3 compared to PBL. Moreover, $CD4^+ CD25^{hi}$ TIL significantly express CTLA-4, TIM-3, and PD-1, but not LAG-3 compared to their PBL counterparts. We also observed that stimulation with anti-CD3/28 (mimicking TCR triggering) upregulated TIM-3 and LAG-3 by TIL and PBL,

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while upregulating CTLA-4 and PD-1 only by TIL. Moreover, the expression level of TIM-3 and PD-1 was higher on TIL isolated from HPV-positive tumors compared to that of HPV-negative tumors, suggesting that HPV-positive tumor-derived antigenic stimulation is one of the key events to upregulate these inhibitory receptors in the HPV-positive tumor microenvironment. Interestingly, blockade of one inhibitory receptor during TCR stimulation upregulated other receptors in a compensatory manner, supporting clinical potential of combination therapies using the blockade of these inhibitory receptors. Regarding the cytolytic phenotypes, $CD8^+$ TIL expressed relatively high level of granzyme B, which was tightly correlated with TIM-3 and PD-1 expression and defective perforin expression. More importantly, immunotherapy of a cohort of HNC patients with the EGFR-specific mAb cetuximab increased perforin and granzyme B expression in both $CD8^+$ PBL and TIL from HNC patients, and upregulated CTLA-4, TIM-3, and PD-1 expression on TIL. Taken together, these findings suggest that CTLA-4, TIM-3, and PD-1 are useful biomarkers to reflect antigenic stimulation status of TIL in the tumor microenvironment, and support cetuximab-based cancer immunotherapy to reverse TIL dysfunction, thus potentially improving clinical outcome of HNC patients.

Key Words: HPV, Tumor infiltration lymphocytes, Tumor microenvironment.

Bone Marrow-derived Stromal Cells (BMSC) Show Both Pro-inflammatory and Immunosuppressive Characteristics in Melanoma Microenvironment: An in vitro Study by Co-culture of BMSC, TIL and Melanoma

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Background: BMSC immunomodulatory effects in tumor microenvironments are poorly defined and controversial; some studies show they promote tumor progression and metastasis while others report they suppress tumor growth. BMSC can modulate immune cells, but their effects are thought to be primarily immunosuppressive and mediated through IFNγ- and TNFα-induced BMSC expression of IDO. Others have shown that BMSC are polarized toward a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype through TLR3 and TLR4 specific priming respectively. We in vitro co-cultured BMSC, tumor-infiltrating lymphocytes (TIL) and melanoma cells to elucidate how the soluble factors within the tumor microenvironment influence BMSC activation in vivo and the effects of activated BMSC on immune cell function.

Methods: Healthy donor BMSC were co-cultured with antigen specific TIL from two HLA-A2 + melanoma patients and two melanoma cell lines (HLA-A2 + or HLA-A2-). TIL and melanoma were co-cultured in physical contact but separated from BMSC by TransWell. Cells and supernatants were harvest after 24 hours, 48 hours and 72 hours. BMSC and TIL gene expression was assessed by microarray (Agilent). The levels of 42 supernatant factors were evaluated by Mutiplex ELISA (Aushon).

Results: TIL gene expression profiling showed IFNγ expression increased 9-fold after co-culture with HLA-A2 + melanoma cells compared to TIL co-cultured with HLA-A2- cells. Gene expression analysis of BMSC co-cultured with TIL and HLA-A2 + melanoma showed marked changes compared with control BMSC. In contrast, the co-culture of BMSC with TIL and HLA-A2- melanoma resulted in far few changes. In fact, Principle Component Analysis (PCA) and clustering analysis could not separate BMSC co-cultured with TIL and HLA-A2- melanoma and control BMSCs. These results show that IFNγ, released by melanoma-activated TIL, plays a critical role in BMSC activation. BMSC co-cultured with TIL and HLA-A2 + melanoma up-regulated both MSC1 and MSC2 genes. MSC1 genes included a vast array of pro-inflammatory factors such as CCL2, CCL5, CCL8, CXCL9, CXCL10, CXCL11, IL6, IL12, IL15, while IDO expression, critical for MSC2 polarization, increased 171-fold.

Conclusion: Melanoma-activated TIL product soluble factors that polarized BMSC towards a MSC1 and MSC2 phenotype. The balance between MSC1 and MSC2 and their pro-inflammatory and immunosuppressive effects may explain the discrepant impact of BMSC on tumors. Further studies are needed to better understand the mechanism responsible for balancing these two phenotypes.

Key Words: Immunomodulation, Tumor microenvironment, Tumor stromal cells.

Tracking Tumor Infiltrating B Cells Revealed Cancer Initiating Cells That Coexpress Unique GD3 Sialilated Glycosphingolipides and CD20 in Metastatic Malignant Melanomas

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Background: Our project aimed to harness the potential capacities of B cells in cancerous tissues in patients with metastatic malignant melanomas. We proved the tumor infiltrating B (TIL-B) cells' unique GD3 ganglioside binding capacity that urged to reveal other functional and regulatory properties of B cells in relation to the tumor environment.

Objectives: We approached the question by tracking TIL-B cells in metastatic malignant melanomas and defined characteristic tumor associated antigens. We aimed to reveal basic components of key regulatory mechanisms in tumorigenesis and metastatic potential with a novel approach.

Patients and Methods: Minor fresh samples from surgically removed cancerous tissues, lymphnode metastases blood (n = 62) of patients with metastatic malignant melanomas. Core biopsies of liver metastases were examined also (n = 31). Immunohistochemistry was performed on tissue microarrays and core biopsies. Fresh cancerous tissue cultures were set up and investigated by immunofluorescence and molecular genetic assays (e.g. Real-time PCR) in terms of characteristic parameters in the tumor microenvironment.

Results: Immunohistochemistry defined a characteristic colocalisation of specific GD3 sialilated glycosphingolipides and CD20 antibody positive areas in primary melanoma tissues and core biopsies, and lymph node frozen tissues. A minor double positive cell population (0.1%) with unique GD3 and CD20 or CD19 positivity could be sorted out of tough cancerous outgrowth. Rescued cell population was investigated for cancer stem cell markers (e.g. CD133, Nestin). Real-time PCR was set up to characterize the minor population and define molecules that play a potential regulatory role.

Conclusion: Our novel approach opens a door for the specific detection and potential elimination of cancer initiating cells in patients with metastatic melanomas. The results give first hint what are those regulation processes, where TIL-B cells might be involved in the tumor microenvironment and which mechanisms tip the balance?

Key Words: Metastases, B cell, Melanoma.

Pancreatic Cancer Associated Stellate Cells Differentiate Immune Cells into an Immunosuppressive Phenotype

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Pancreatic stellate cells (PSC), also known as cancer associated fibroblasts, can provide pro-survival signals to tumor cells, however their interactions with immune cells within the tumor microenvironment have not been explored in detail. In this study, we hypothesized that soluble factors produced by PSC can promote immunosuppression within the pancreatic tumor microenvironment by enhancing myeloid-derived suppressor cell (MDSC) differentiation and recruitment. PSC were isolated from fresh tissue of patients undergoing surgical resection for pancreatic adenocarcinoma at the OSUCCC. PSC grew out of the tissues within 5-7 days. Fibroblast morphology was confirmed via positive staining for vimentin, alpha-SMA, and GFAP and analyzed by fluorescence microscopy. Supernatants were collected when PSC cultures were ~70% confluent and assessed for chemokine and cytokine inflammatory mediators by ELISA or Bioplex analysis (Affymetrix). PBMC obtained from normal healthy donors (American Red Cross) were cultured in the presence of 5 and 10% PSC supernatants or stimulated with IL-6/GM-CSF (positive control) for 7 days. Cells were then assessed for MDSC phenotype by flow cytometry. To date, we have generated separate primary PSC stellate cell lines from patients (n = 7). Bioplex and ELISA analysis indicated that these PSC produce numerous pro-inflammatory cytokines including IL-6 (300-650 pg/mL), VEGF (700-2000 pg/mL) and MCSF (150-200 pg/mL). We investigated whether PSC could induce the differentiation of immune cells into an MDSC phenotype. Indeed, PBMCs incubated in the presence of PSC supernatants for 7 days differentiated into cells with an MDSC phenotype (mean of 13.75 ± 2.47% of cells CD11b + CD33 + , $P < 0.05$). We confirmed that the CD11b + CD33 + cells generated in the presence of PSC could suppress autologous T cells stimulated with CD3/28 activation beads (80 ± 22% inhibition). Culture of normal donor PBMCs with PSC supernatants also resulted in STAT3 Tyr705 phosphorylation as determined by immunoblot analysis. The PSC-mediated MDSC differentiation was STAT3-dependent as culture in the presence of FLLL32, a small-molecule STAT3-specific inhibitor, significantly inhibited PSC-induced MDSC differentiation ($P < 0.05$). To our knowledge, these findings represent the first report of human pancreatic stromal cells as modulators of MDSC differentiation. These data also define potentially mechanisms of cellular cross-talk within the pancreatic tumor microenvironment. We are hopeful that these findings may uncover novel cellular or molecular mediators that can be manipulated to treat this deadly disease.

Key Words: Immunosuppression, Tumor microenvironment, Tumor stromal cells.

AKT + β-CAT Liver Tumor Development is Dependent on B Cells

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Acute liver inflammation is a necessary response to liver damage or infection. However, the effects of chronic liver inflammation, manifesting as liver fibrosis and cirrhosis, are sufficient in a fraction of individuals to induce deleterious mutations igniting transformation and hepatocellular tumors. Tumorigenesis is a dynamic process that involves a combination of initiating-oncogenic mutations and complex contributions from host stromal and infiltrating immune cells. By contrasting tumor development in wildtype (WT) and specific immune-deficient mice, this study identifies a significant role for B cells in the promotion of oncogene-induced tumor development in the liver. We initiated tumorgenesis in the liver, by hydrodynamically injecting Sleeping Beauty constructs expressing mutated forms of AKT and beta-catenin, two genes frequently dysregulated in liver cancers, along with gaussia luciferase which allows us to monitor tumor development. Interestingly, tumor progression was markedly reduced and survival was significantly enhanced in RAG1-/- and B cell-/- (Igh6-/-) mice, as compared to WT, CD4-/- and CD8-/- C57/BL6 mice.. Moreover, expression analysis of CD45 + CD19 + sorted B cells from AKT + CAT induced hepatic tumors revealed elevated levels of TNFα, LTβ, and Light. Subsequent co-delivery of AKT + CAT into TNFα/LTα/β-/- and TNFR1-/- mice resulted in similar, significant decrease in tumor progression and increased survival. Additionally, chronic treatment with agonistic anti-LTβR clone 4H8 led to a complete restoration of tumor development in B cell-/- mice. Mechanistic studies are underway investigating the dependence of B cell-derived lymphotoxin during AKT + CAT initiated hepatic tumor development. This de novo approach provides insight into novel intersections between specific oncogenic pathways and host immune responses.

Key Words: B cell, Tumor microenvironment.

New Technologies for Measurements of Tumor Infiltrating Lymphocytes

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The presence, abundance, population, and diversity of Tumor Infiltrating Lymphocytes (TILs) have been identified as prognostic indicators in several immunogenic cancers. However, current methods to study TILs are largely limited to flow technologies to count and identify T cell type. Emerging cancer therapeutics including immunomodulators and adoptive T-cell therapy highlight the need to better understand and track this population of T cells. We've developed two complimentary amplification based high-throughput methods to characterize the adaptive immune response to a tumor. Both methods utilize a multiplex PCR to amplify rearranged T-cell receptor Beta (TCRB) chains; one method uses droplet digital PCR (ddPCR) to count T cells while the other uses high-throughput sequencing (HTS) to characterize the immune repertoire. The majority of TCR diversity resides in the β chain, and each T cell clone expresses a single TCRB allele that has been rearranged from the germ-line TCRB locus to form one of many billions of possible TCRB genes. This immense diversity is generated by combining noncontiguous variable, diversity, and joining region gene segments, which collectively encode the CDR3 region and determine antigen specificity. This occurs after T cell lineage commitment and rearranged TCRB CDR3 chains are unique to T cells, so the number of rearranged chains is directly correlated with the number of αβ T cells. In our ddPCR assay, we use multiplex PCR primers and fluorescently labeled probes that specifically anneal to V genes to count T cells using the QuantaLife Droplet Reader. Our sequencing assay uses the Illumina system to sequence across the CDR3 region along with our previously developed bioinformatics tools to identify the diversity of T cell clones based on CDR3 sequence. In concert, these two assays can both count the number and characterize the repertoire of αβ T cells in a given tumor sample. To show utility, we use our assays to characterize the heterogeneity of ovarian tumor TIL populations. We apply our assays to 10 primary and metastatic ovarian tumors collected from 5 patients. Each tumor is divided into a grid pattern with 8-10 sections and two samples are collected from each grid. DNA is isolated from each sample, and from each sample a subset of DNA is used for the ddPCR and the rest is used for HTS. For each tumor section we collect data on the number, diversity, and the unique CDR3 sequences carried by the TILs. We use these data to characterize the intra-tumor heterogeneity of TIL count, diversity, and T cell clone overlap. We find that within a tumor, adjacent samples show greater similarity to each other suggesting that the TCR repertoire of the tumor environment is heterogeneous.

Key Words: Ovarian cancer, Tumor infiltration lymphocytes, Tumor microenvironment.

The Immune-related Role of BRAF Mutation in Melanoma

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Malignant cutaneous melanoma is an aggressive neoplasm characterized by a complex etiology that challenges targeted therapies. The most commonly mutated pathway is the mitogen-activated protein kinases (MAPK) cascade. The activation of the MAPK pathway occurs through gain-of-function mutations in the BRAF and NRAS genes.

Although the oncogenic potential of BRAF and NRAS alterations has been related to a reduced apoptosis, increased invasiveness and increased metastatic behavior, the role of BRAF and NRAS in the immunological landscape of cutaneous melanoma has been poorly investigated. It is now emerging the existence of at least two different immune phenotypes in melanoma, a Th17 phenotype associated with over-expression of WNT5A, enhanced cellular motility and poor prognosis, and a Th1 immune phenotype associated with a more differentiated status and better prognosis.

However, it is not clear yet whether these two different phenotypes depend upon the genetic background of the host, of the tumor or of both.

Here, we tested whether the Th1 and Th17 phenotypes could be at least in part explained by BRAF and NRAS mutations in melanoma. 113 pre-treatment snap frozen tumor biopsies were collected from patients treated at the Surgery Branch, National Cancer Institute, Bethesda, Maryland. BRAF and NRAS mutational status were assessed by capillary sequencing. RNA was isolated and processed for microarray analysis (Affymetrix).

Among the 113 metastases, 59% and 12% were mutated in BRAF and NRAS respectively. When assessing genes concordantly deregulated in BRAF and NRAS mutant samples, many of them resulted to encode constituents or regulators of the MAPK/ERK and related pathways.

When performing class comparison between BRAF mutant and wild-type samples, metastases showing a Th17 phenotype were preferentially BRAF mutated. Moreover, some of the genes differentially expressed between BRAF mutant and wild-type samples resulted to be involved in immune-related pathway (IL-1, IL-17 and IL-15 pathways) and, most importantly, they were discriminative of the Th1 and Th17 immune phenotypes in the 113 melanoma metastases.

In contrast, genes differentially expressed between NRAS mutant and wild type samples were not discriminative of Th1 and Th17 phenotypes.

These findings have important implications for combined BRAF targeted therapy plus immunotherapy for melanoma.

Key Words: Melanoma.

The Ratio of Co-cultured CD14 + Monocytes and Tumor Cells Influences Loss of HLA-DR on Monocytes

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Background: Antigen presentation is a vital function of monocytes which are characterized by high expression of MHC Class II HLA-DR. Previous work in our lab has shown an increased ratio of CD14 + HLA-DRlow/- cells in the peripheral blood of cancer patients. In addition, the presence of these cells correlates with increased systemic immunosuppression, a pro-angiogenic environment and increased number of monocytes within the tumor. We hypothesize that the loss of HLA-DR may be a universal phenomenon associated with malignant cells interacting with CD14 + cells in-vivo, leading to a conversion of these cells into an immunosuppressive and tumor-protective phenotype of CD14 + HLA-DRlow/- cells. The mechanism(s) for the tumor mediated loss of HLA-DR and other associated phenotypic changes of monocytes during co-incubation of tumor is unknown.

Objectives: To identify tumor mediated HLA-DR changes in CD14 + cells.

Methods: Fresh CD14 + monocytes from healthy donor blood samples were collected and immunophenotyped for baseline characteristics. CD14 + cells were co-cultured with a renal cancer cell line (ACHN) at incremental proportions (ACHN cell:CD14 + cells at 1:2, 1:8, 1:32) for 4 days in advanced DMEM with 5% Fetal bovine serum medium. The same was repeated with a separate sample where ACHN and CD14 + cells were co-cultured at 1:64 ratio for 4 days. Co-cultured cells were collected after 4 days for flow cytometry to measure cell surface markers. Data was collected on a BD FacsCalibur, and analyzed using FlowJo 7.6 and GraphPad Prism software.

Results: Loss of HLA-DR in the CD14 + cells was observed consistently after co-culture with renal cancer cells for 4 days. This effect was significant when compared to baseline HLA-DR expression on fresh healthy donor CD14 + cells ($P < 0.05$). CD14 + cells consistently lost HLA-DR independent of tumor to CD14 + cell ratios.

Conclusions: The in-vivo phenomenon of loss of HLA-DR on CD14 + cells was successfully replicated in vitro by tumor co-culture with CD14 + monocytes. Tumor cells (ACHN cells in this particular experiment) interact with CD14 + HLA-DR + monocytes and convert them into CD14 + HLA-DRlow/- immunosuppressive cells. However, the effect seems to extend beyond a simple model of tumor and monocyte interaction. The decrease in HLA-DR levels at higher tumor to monocyte ratios suggest interaction amongst CD14 + cells promoting further change to immunosuppressive phenotype. This observation is clinically significant as the CD14 + HLA-DRlow/- cells have been shown to be an independent unfavorable prognostic factor in cancer patients and therefore a potential therapeutic target.

Key Words: Immunosuppression, Tumor microenvironment.

TUMOR VASCULATURE, CHEMOKINES AND LYMPHOCYTE TRAFFICKING TO THE TUMOR

Immunological Phenotyping of Colorectal Carcinoma Liver Metastases and Primary Ovarian Cancer

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Introduction: Characterizing TIL' s composition allows monitoring of immunotherapies and has been correlated to patients outcome. Here we analyze phenotype and function of lymphocytes collected from peripheral blood (PBL) and tumor infiltrating lymphocytes (TIL) from patients with two different types of cancer and two different tumor sites: colorectal cancer metastases (CRLM) and ovarian cancer primary tumors (OVC).

Methods: 14 CRLM and 18 OVC samples were collected in the operating room immediately following resection. Samples were analyzed using a multi-color flow analysis panel containing CD3, CD4, CD8, CD25, CD38, HLA-DR and the intracellular markers, FoxP3 and Ki-67. Cytokine production from purified PBL and TIL CD4 + T cells was analyzed by RT-PCR.

Results: In CRLM patients, there was no difference in the percentage of Tregs (CD4 + /CD25 + /FoxP3 +) in PBL and TIL: 8.6% versus 11%. We did find an increased frequency of Tregs in the primary OVC TILs when compared to PBL: 21.3% versus 4.9% ($P = 0.003$). A subpopulation of highly suppressive Tregs expressing HLA-DR was markedly increased in both TIL compared to PBL, CRLM: 67.9% versus 37.1% ($P = 0.0002$) and OVC: 73% versus 37% ($P = 0.003$). The cytokine profile showed that IL-6, a cytokine creating an immunosuppressive environment, is uniquely detected in TIL samples. Both TIL populations also contained a significantly higher

proportion of activated cytotoxic CD8 + T cells (HLA-DR + / CD38 +) compared to PBL (CRLM: 30.8% vs. 7.7%, ($P < 0.01$), OVC 53% vs. 10%, ($P = 0.002$)). The frequency of CD4 + FoxP3-CD25int T cells, potentially representing a subset of memory T cells responsive to recall Ag, was decreased in both tumors TIL compared to PBL: CRLM, 11.3% versus 35.7% ($P = 0.003$) and OVC, 11.8% versus 32.8% ($P = 0.0003$).

Conclusion: This study demonstrates that multi-color flow of fresh tumor samples is an effective method to study phenotype and activation state of lymphocytes within hours of resection and may reveal populations not seen in fixed tissue samples. TIL composition in primary and metastatic tumors from two different organs is remarkably similar, with a higher proportion of highly suppressive Tregs (HLA-DR +) and activated cytotoxic T cells (CD8 + /HLA-DR + /CD38 +). We also found a significant decrease in the percentage of memory T cells within tumors versus peripheral blood (CD4 + /FoxP3-/CD25int). It is essential to identify these populations in order to optimize and track results of immune-based therapy.

Key Words: CD8 + T cells, Treg cells, Tumor infiltration lymphocytes.

Treatment of Melanoma and Endothelial Cell Lines With TLR Agonists Alters Immune Activating Cytokine Production

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Despite efforts to eradicate melanoma by active immunization or adoptive T cell transfer, immune-mediated cancer regression occurs in a small minority of patients. Newer strategies to overcome tumor induced immune suppression involve altering the activation and homing abilities of immune cells. Chemokines are cell-secreted proteins that function to recruit immune cells to sites of infection or damage. Toll-like receptors (TLRs) are known to be expressed on epithelial cells, and TLR agonists have been demonstrated to influence the activity of dendritic cells (DC) and DC-induced immune responses. Therefore, we hypothesized that melanoma and endothelial cell lines may also express TLRs, and that treatment with TLR agonists would alter the chemokine expression of these cells to better facilitate immune cell homing and activation. Moreover, we hypothesized that TLR agonist treatment in combination with IFNα or IFNγ stimulation would further enhance chemokine secretion by melanoma and endothelial cell lines.

To test these hypotheses we treated several melanoma and endothelial cell lines individually with TLR agonists and analyzed the induced-cytokine expression by flow cytometric analysis. The following TLR agonists were tested: LPS (TLR4), CpG (TLR9), Resiquimod (TLR7/8), Imiquimod (TLR7), and poly-ICLC (TLR3). We found that TLR treatment alone had modest effects on expression of chemokines CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL12 by human melanoma cell lines VMM1, DM13, DM93, and DM122, and human endothelial cell lines HUVEC and HMVECad. However, when melanoma cell lines were treated with TLR agonists + IFNα cytokine expression of CXCL12 was diminished. Conversely, treatment of endothelial cell lines with TLR agonists + IFNγ significantly increased the expression of CCL2, CCL3, CXCL9, CXCL10 and CXCL12.

Together these data indicate that TLR agonist treatment in combination with IFNα or IFNγ alters the chemokine expression of endothelial and melanoma cell lines, which may ultimately promote stronger tumor-immune recognition and responsiveness. DCs are widely considered the canonical TLR-expressing cell, but these data show that endothelial and melanoma cells also respond to TLR agonists and likely also impact the metastatic melanoma microenvironment (MME). Our data show that in response to TLR agonists + IFNγ treatment melanoma cell lines displayed minimal changes in chemokine production, while endothelial cells responded with increased expression of several immune modulating chemokines. Therefore, these data indicate that in vivo IFNγ production by Th1 cells may augment the responsiveness of other cells, including endothelial and melanoma cells to TLR agonists.

Key Words: Chemokines, Melanoma, Tumor microenvironment.

Cross-regulation of NF-κB-driven Chemokine Production by STAT-1/IRF1 Versus PKA/PCREB Pathways Determines the Ability of Tumor Microenvironment to Preferentially Attract Effector Versus Regulatory Cells

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Tumor infiltration with effector CD8 + T cells (Teff) predicts longer recurrence-free survival in many types of human cancer, while local accumulation of regulatory T (Treg) cells is a negative prognostic factor. Using colorectal tumor explants and isolated subsets of tumor-associated cells, we show that IFNs and PGE2 are the dominant determinants of tumor-associated production of Teff and Treg chemokines, mediating their effects through STAT1/IRF1- and PKA/pCREB-mediated modulation of the with NF-κB-driven chemokine production. In different individual tumor explants, we observed highly heterogeneous responses to IFNα or poly-I:C (a TLR3 ligand) when they were applied individually. In contrast, a combination of IFNα and poly-I:C uniformly enhanced the production of CXCL10/IP10 and CCL5/RANTES (Teff-attracting chemokines) in all tumor lesions. Addition of COX inhibitors to the combination of IFNα and poly-I:C, further enhanced these desirable effects and uniformly suppressed the production of CCL22/MDC, a chemokine associated with infiltration of T regulatory cells (Treg). The Teff-enhancing effects of this treatment occurred selectively in tumor tissues, as compared to tissues derived from tumor margins. These effects relied on the increased propensity of tumor-associated cells (mostly fibroblasts and infiltrating inflammatory cells) to hyper-activate NF-κB and produce Teff-attracting chemokines in response to treatment, resulting in an enhanced ability of the treated tumors to attract Teff cells and reduced ability to attract Tregs. Together, our findings suggest the feasibility of exploiting NF-κB hyper-activation in the tumor microenvironment to selectively enhance Teff entry into colon tumors.

Key Words: Chemokines, Tumor infiltration lymphocytes, Tumor microenvironment.

IL-18-primed "Helper" NK Cells Mediate the Attraction and Activation of DCS, Promoting the Accumulation of Type-1-effector T Cells at Tumor Sites

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The chemokine-driven interaction of immune cells is essential for effective anti-tumor immunity. Natural killer (NK) cells can be primed by IL-18 for unique "helper" function, promoting dendritic cell (DC) activation and DC-mediated induction of type-1 immune responses against cancer. We demonstrate that such IL-18-treated "helper" NK cells are selectively primed for high expression of the immature DC (iDC)-attracting chemokines CCL3 and CCL4 upon subsequent exposure to accessory cell signals, including type I interferons, IL-15, IL-12, or IL-2. These "helper" NK cells potently attract iDCs in a CCR5-dependent mechanism and induce high DC

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production of the CXCR3 and CCR5 ligands CXCL9, CXCL10, and CCL5, facilitating the recruitment of type-1 effector T (Teff) cells to tumor sites. Using cells isolated from the malignant ascites of patients with advanced ovarian cancer, we demonstrate the therapeutic potential for using "helper" NK cell-inducing stimulatory factors to enhance Teff cell-recruiting chemokines directly within the human tumor environment. This study demonstrates for the first time the unique chemokine expression associated with "helper" versus "killer" NK cell differentiation, and provides rationale for the therapeutic use of properly-activated NK cells in promoting type-1 immune responses against cancer.

Key Words: Chemokines, Dendritic cell, NK cells.

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