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(Primary Authors are Italicized)

ADOPTIVE T-CELL TRANSFER: THE NEXT WAVE

Development of a γ-Retroviral Vector for the Expression of Artificial miRNAs in Human T Lymphocytes

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Since its discovery, RNA interference has not only emerged as a new field of study but also provided a series of versatile tools for the study of a wide range of biological processes. While chemically synthesized short interfering RNAs (siRNA) and short hairpin RNAs (shRNA) expressed under RNA Pol III promoters have been satisfactorily utilized in basic genomic studies, their widespread application in therapeutic settings is still hampered by technical issues. Although the necessity of repeated administration of siRNAs had been overcome by using vector-encoded shRNAs, it has been reported that these may induce toxicity due to saturation of host cell processing machinery. To address this, chimeric structures that mimic endogenous miRNAs, termed artificial miRNA (amiRNA), have been developed based on mir-30 and mir-223 backbones. In this work, we sought to develop a gammaretroviral (RVV) vector to conjointly express a transgene and amiRNAs based on miRNAs naturally expressed in human lymphocytes. A set of five amiRNAs were synthesized that contain the sequence of either mir-142, mir-155, mir-223, mir-146b and mir-150 primary transcripts, in which each wild type stem region has been replaced by a control sequence with no specificity for any human gene. Each of these chimeric contructs was cloned into BglII or BsrGI sites located in the intronic region of pMSGV2-tCD34 plasmid, which encodes for a truncated form of CD34 surface marker that was used as a reporter gene. All ten constructs were used to generate RD114-pseudotyped RVV that were tested, by flow cytometry, for their ability to induce tCD34 expression in transduced human PBL. This parameter was used to select the most efficient combinations of miRNA backbone and cloning site, in terms of transgene expression. Thus, mir-142-, mir-155- and mir-150-derived amiRNAs yielded the highest percentage of CD34 positive cells. Of note, vectors expressing mir-223-based amiRNAs induced almost negligible expression of transgene, as opposed to previous reports using lentiviral vectors, stressing the relevance of vector-specific step-wise optimization. This platform may allow for the validation of gene silencing as an enhancer of T cell activity, as well as a delivery vehicle for tumor-specific T cell receptors (TCR) or Chimeric Antigen Receptors (CAR) genes concomitantly with silencing molecules in the context of adoptive cell therapy.

Depletion of NK Cells Enhances the Effectiveness of Adoptive Cell Therapy with Naive Tumor-specific CD4+ T Cells Through Surface Bound IL-15

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CD8+ T cells have been the primary focus of immunotherapy of cancer with little focus on CD4+ T cells. Immunotherapy involving in vitro differentiated T cells given after lymphodepleting regimens significantly augments antitumor immunity in animals and human patients with cancer. However, the mechanisms by which lymphopenia augments adoptive cell therapy are still emerging. We demonstrate that naïve tumor/self-specific CD4+ T cells naturally differentiated into T helper type 1 (Th1) cytotoxic T cells in vivo and caused the regression of established tumors and depigmentation in lymphopenic hosts. Therapy was independent of

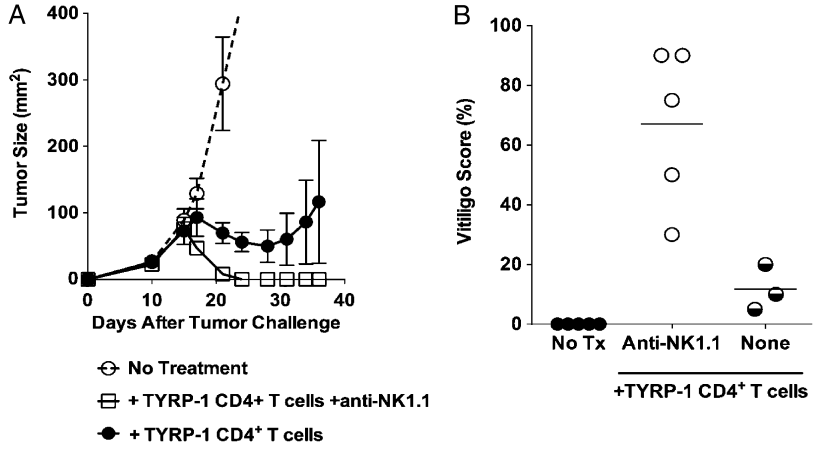

FIGURE 1. A, Depletion of NK cells enhances adoptive cell therapy of established melanoma with naive tumor/self-specific CD4+ T cells. B, Enhanced autoimmune vitiligo with NK depletion and adoptive cell therapy with naive tumor/self-specific CD4+ T cells.

vaccination, exogenous cytokine support, CD8+-, B-, NK and NK T cells. Surprisingly NK depletion further enhanced tumor therapy by increasing T cell numbers, autoimmune vitiligo, and serum proinflammatory chemokines and cytokines. This effect may be dependent on increased levels of surface-bound IL-15 on CD11c+ MHC class II+ cells which occurs after NK depletion. These data show a potential role for IL-15 in enhancing antitumor therapies which incorporate CD4+ T cells (Fig. 1 previous page).

Evaluation of CXCR3 and CCR5 Polymorphisms and Gene-Expression as Predictive Biomarkers of Clinical Response to Adoptive Therapy in Melanoma Patients

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Background: Adoptive cell therapy induces objective responses in approximately 50% of patients with metastatic melanoma. The recruitment of T lymphocytes through CXCR3, CCR5-ligand chemokines is critical for the development of immune-mediated rejection. A common single nucleotide polymorphism of CXCR3 (rs2280964) has been associated with variation in chemotactic activity. CCR5 polymorphism Δ32 (a deletion of 32 bases encoding a protein not expressed on cell surface), has been recently correlated with poor prognosis in metastatic melanoma patients. We postulated that polymorphisms of CXCR3, CCR5 genes may influence the migration of tumor-infiltrating lymphocytes (TIL) on tumor site and, eventually, the tumor regression.

Methods: One-hundred-forty-two TIL samples, belonging to 142 melanoma patients enrolled in consecutive adoptive therapy trials, were evaluated. Genotyping (rs2280964, Δ32 mutation) was performed by sequencing. Gene-expression profiling of infused TIL was assessed by Affymetrix Human Gene ST 1.0 array (CXCR3, 27 probes; CCR5, 30 probes). DNA/RNA data were correlated with each other, with clinical response.

Results: No significant correlations between genotype, gene expression were detected, neither between CXCR3 polymorphisms, response. Surprisingly, CCR5Δ32 carriers (N = 25; heterozygous, N = 24; homozygous, N = 1) had a better overall response (OR: CR, complete remission or PR, partial remission) compared to wildtype patients (OR: 68% vs. 46%, respectively, $P < 0.05$). The under-expression of CXCR3, CCR5 genes was independently correlated with CR ($P < 0.05$). Interestingly, the co-underexpression of both genes was even more accurate in the prediction of CR: 28% (11/39) in CXCR3CCR5-low group compared to 5% (5/103) in the other patients ($P < 0.0001$). Moreover, the protein-prediction model was also predictive of OR: 64%(28/44) in CXCR3CCR5-low group, 44% (43/98) in the other patients ($P < 0.05$). In this model CCR5Δ32 carriers with high CCR5, low CXCR3 transcript levels were included on the CXCR3CCR5-low group.

Conclusions: CCR5, CXCR3 transcript under-expression in TIL is associated with CR. The protein-prediction model suggests also a correlation with OR. This unexpected result allows to generate new hypotheses on the role of these pathways in the modulation of stimulatory or regulatory mechanisms in different conditions.

Development of HLA-A2 Restricted TCR Against Cancer Testis Antigen SSX-2 for Adoptive Immunotherapy of Cancer

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The expression of cancer testis antigens (CTA) is restricted to tumor cells and non-MHC expressing germ cells of testis, thus they are attractive targets for adoptive immunotherapy. Synovial sarcoma X (SSX) breakpoint proteins are encoded by a family of 10 homologues genes SSX: 1 to 10 located at chromosome Xp11.23. SSX-2 is expressed in a significant proportion of human cancers including in melanomas, head and neck cancers, lymphomas, multiple myeloma, pancreatic cancer, prostate cancer, sarcomas, hepatocellular and colon carcinomas, but not in normal tissues. Therefore, SSX-2 is a promising target for T cell based adoptive immunotherapy of cancer. In an effort to develop TCRs to engineer peripheral blood lymphocytes (PBL) for tumor therapy, we isolated four HLA-A2 restricted TCRs from natural T cell clones generated from tumor-infiltrated lymph nodes from two melanoma patients seropositive for SSX-2 and whose tumor expressed SSX-2. Retroviral vectors were constructed expressing TCR α and β chains incorporating 2A ribosomal skip peptide. PBL were transduced with SSX-2 TCRs and tested for peptide reactivity and tetramer binding. PBL engineered with three of the four TCRs showed SSX-2: 41 to 49 (KASEKIFYV) peptide specific reactivity, tumor cell recognition and tetramer binding. One of the TCR (TRAV14/DV*01, TRBV15*02-CB1) exhibited tetramer binding in both CD4 and CD8 cells, and this TCR was selected for further development. Analogous peptides homologous to SSX-2: 41 to 49 are present in the other members of SSX family of proteins (differing by only 1 or 2 amino acids) and peptide epitopes derived from SSX-3, 4, 5, 9 and 10 were recognized by the SSX-2: 41 to 49 TCR in co-cultures assay with peptide pulsed T2 cells at concentrations of > 10 ng/mL while SSX-2 peptide was recognized at < 10 pg/mL. High levels of antigen specific interferon-γ (IFN-γ) secretion was observed following culture of TCR engineered PBL with several SSX-2+/HLA-A2+ tumor cell lines including melanoma (938-A2 mel, SK MEL 37, T567A, 624, 1300), glioma (U251), ovarian carcinoma (SKOV3), 293-A2 and COS-A2 cells that were retrovirally transduced with SSX-2 gene. Co-culture of TCR engineered PBL with SSX-2- and/or HLA-A2- cell lines produced only background levels of IFN-γ. Based on these results, this TCR may be an attractive candidate for potential cancer gene therapy.

In Vitro Generation of Multiantigen-Specific CD4+ and CD8+ T Cells for Adoptive Immunotherapy

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Adoptive transfer of T cell populations with specificities for several antigens is a promising strategy to treat cancer or prevent multiple infections in immunocompromised patients. Here, we describe a protocol to generate multivirus-specific T cells, using pools of peptides for in vitro T cell stimulation followed by magnetic enrichment of functional T cells according to IFN-γ secretion. Peptide pools consist of synthetic peptides with 15 amino acids (aa) lengths and 11 aa overlap covering the complete antigenic protein. In short-term in vitro stimulations of PBMC from healthy donors we compared peptide pools with recombinant proteins and immunodominant peptides to induce an IFN-γ response in specific T cells. Using the CMV antigens pp65 and IE-1 we found both, peptide pools and recombinant proteins, efficiently activate CD4+ T cells. In contrast, efficient stimulation of CD8+ T cells was achieved only using either the peptide pools or the immunodominant peptides. To test the usability of peptide pools to obtain multivirus-specific T cells for adoptive therapy we stimulated PBMC of healthy donors with a combination of 4 selected peptide pools covering CMV pp65 or IE-1, adenovirus hexon, EBV EBNA-1 or BZLF-1 for 4 hours. Concomitant addition of 4 antigens to a single sample might decrease the activation efficiency for each specific T cell due to competition of peptides for MHC binding. As a control, we split PBMC into 4 aliquots, incubated each sample

with a single peptide pool for 2 hours, and recombined for another 4 hours. The IFN-γ Secretion Assay was used to magnetically enrich IFN-γ secreting T cells to a purity of > 90%. High antigen specificity and functionality of the enriched T cells was confirmed after expansion for 9 to 14 days. T cell lines contained high frequencies of pp65tetramer+ CD8+ T cells. Additionally, after restimulation with a mixture of peptide pools 21% to 53% of CD4+ T cells and 53% to 87% of CD8+ T cells produced IFN-γ. Moreover, comparing T cell frequencies specific for each single antigen in PBMC with that in multivirus-specific T cell line after separate reactivation with peptide pools by analysis of IFN-γ production, we observed the specificity for each antigen sustained after enrichment and expansion. We found similar results in number of enriched cells, expansion rates, and antigen-specificity of the T cell lines, regardless of whether PBMC loaded with a mixture of antigens or separately with single peptide pools to generate T cell lines. In summary, we established a protocol for rapid in vitro generation of multiantigen-specific CD4+ and CD8+ T cells using a combination of peptide pools from several antigens for restimulation and subsequent magnetic selection of IFN-γ secreting T cells.

Hypotheses for Improving Designer T Cells; Phase I Trials

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In prior Phase I trials of 1st generation (1st gen) designer T cells, proof-of-principle ''biologic responses'' were noted, but with poor in vivo persistence and transient in-tumor activity. The first problem generated the hypothesis: If T cells were maintained systemically at high levels, a sustained T cell percolation into tumor could yield cures even if T cells survived for only a few days of killing. The second problem generated the hypothesis: If T cells entering tumor, though few in number, were to proliferate on contact with antigen, then tumors could be cured. In separate clinical trials, these hypotheses are being addressed in our group.

Methods: Patient T cells are transduced and expanded ex vivo to span dose levels of 10^9, 10^10 and 10^11 T cells. The first study uses 1st gen designer T cells (against prostate specific membrane antigen, PSMA), but with prior non-myeloablative (NMA) conditioning to create a ''hematopoietic space'' into which designer T cells are engrafted for prolonged in vivo persistence, with co-administered continuous infusion IL2. The second study uses a simple infusion protocol that applies 2nd gen designer T cells (against CEA) with added CD28 co-stimulation with the goal of in situ amplifying the effector cells that reach tumor.

Results: Each study treated five patients to date at the low 10^9 and middle 10^10 T cell doses. NMA conditioning led to successful T cell engraftment in the 1% to 20% range at one month, versus designer T cells being undetectable at the same time in the infusion study. No on-target T cell toxicities were noted in either study. In the engraftment protocol, two patients had PSA reductions of 50 and 70% in the two months following treatment. In the second study with 2nd gen anti-CEA T cells, one gastric cancer patient had shrinkage of lung and brain mets and another has stable disease for 12+ months after treatment; future patients will have IL2 added with the next T cell dose escalation. The clinical (non-manufacturing) cost per patient was estimated at $60 to 100 K in the engraftment study and $5 to 10 K in the infusion study.

Conclusion: Parallel approaches are being applied to test hypotheses of benefit of engraftment versus benefit of co-stimulation to create effective anti-tumor activity in designer T cells. Study results support the safety of the protocols with indications of efficacy but the dose escalations are at an early stage. Patient recruitments are continuing.

Functional Reprogramming of the Tumor Stroma by IL-12 Engineered T Cells is Required for Anti-tumor Immunity

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Bone marrow derived stromal cells within the tumor microenvironment are capable of cross presenting antigens to cytotoxic T lymphocytes (CTL). We found that the adoptive transfer of tumor-specific CD8+ T cells gene-engineered to secrete IL-12 led to the increased local infiltration of adoptively transferred T cells and caused the regression of large established B16 melanomas. The autocrine effects of IL-12 resulted in the production of large amounts of IFN-γ by T cells. Surprisingly, we found that IL-12-engineered T cells that lacked the ability to receive signals from IL-12 (Il12rβ2 −/−), and indeed T cells that lacked the ability to produce IFN-γ (Ifnγ −/−), retained all of their ability to trigger tumor destruction. However, tumor treatment efficacy was abrogated when the cells in host mice lacked IL-12 receptors (Il12rβ2 −/−), IFN-γ receptors (IfnγR −/−) or the ability to produce IFN-γ. Thus, sensitization of host cells and not the transferred T cells within the tumor microenvironment was critical for successful anti-tumor immunity. We measured increased endogenous CD8+ and host NK cells within the tumor but treatment responses remained robust in mice completely devoid of T and B cells (Rag −/−) and depleted of NK cells. We found that the majority of cells expressing the IL-12Rβ2 receptor within the tumor were CD11b+ myeloid cells. Transfer of IL-12-producing anti-tumor T cells triggered significant in situ changes in CD11b+ cells including increased expression of H-2Db along with up-regulation of Fas (CD95), and FADD. In addition, both the numbers and percentages of CD11b+ cells dropped just prior to tumor regression. Tumor treatment was abrogated in mice deficient in MHC class I (β2M −/−) but not class II (I-Ab −/−), indicating the functional importance of antigen cross-presentation in vivo. These results are consistent with a model whereby IL-12 triggers the functional maturation of in situ APCs capable of cross-presenting tumor antigens. Licensed recognition of these antigens by tumor-specific T cells may in turn trigger the collapse of the tumor stroma and its vasculature.

Monomeric Designer T Cells Kill IL13Ra2 Expressing GBM More Efficiently

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GBM is a devastating primary brain tumor for which there is no effective therapy to specifically target tumor cells. Targeted immuno-gene therapy has been shown effective in a number of tumor models without significant toxicity. We aim to develop potent GBM-specific T cells as an innovative and unique targeted immuno-gene therapy. I exploit IL13Ra2 as a GBM-specific tumor antigen due to its frequent overexpression on a majority of GBM but not on normal brain tissues. Targeting IL13Ra2 on GBM has a strong rationale supported by clinical development of IL13 immunotoxin molecules. IL13 binds two types of receptor with different affinities: IL13 binds to GBM-associated IL13Ra2 with high affinity (Kd = 0.25 to 1.2 nM); IL13 binds to IL13Ra1 first with low affinity (Kd = 2 to 30 nM) and then recruits IL4Ra to the complex. Previous studies showed that substitutions at three sites of IL13 (Glu-11, Lys-103, or Arg-107) proved to be critical for IL13 binding to IL13Ra2, and modifications of these sites can neutralize or dramatically increase the affinity of IL13 to IL13Ra2, compared of the shared IL13Ra1, which is expressed on the normal brain cells and other tissues. In order to achieve both specific targeting and selectively enhanced cytotoxicity of GBM-associated

IL13Ra2, I generated IL13.E11 K.R107 K (both glutamic acid at position 11 and arginine at 107 changed to lysine). I modified T cells using retroviral gene transfer to express a chimeric immune receptor (CIR) comprising an extracellular high affinity mutant IL13.E11 K.R107 K molecule linked to intracellular signaling components from CD3 zeta chain and CD28 co-stimulatory molecule. The IL13.E11 K.R107 K designer T cells show specific targeting and selectively enhanced cytotoxicity of IL13Ra2+ glioblastoma cells, and inhibit tumor growth employing a glioma xenograft model established with human glioma cells in athymic nude rats. I hypothesized that monomeric designer T cells kill IL13Ra2 expressing GBM more efficiently based on the monomeric IL13Ra2 chain is overexpressed on a majority of GBM. I generated monomeric CIR by mutating both Cys-29 of the CD8a hinge and Cys-2 of CD3 transmembrane to Ala. Monomeric designer T cells show specifically enhanced cytotoxicity/INF-g and IL-2 cytokine secretion of IL13Ra2+ glioblastoma cells versus control dimeric designer T cells. Monomeric IL13.E11 K.R107 K designer T cells show the most potent against IL13Ra2+ glioblastoma cells. We demonstrated the in vivo antiglioma efficacy of the monomeric IL13.E11 K.R107 K designer T cells employing a glioma xenograft model established with human glioblastoma cells in athymic nude rats. The monomeric IL13.E11 K.R107 K designer T cells have significant potential for the treatment of recurrent GBM.

Development of a Chimeric Antigen Receptor for Prostate Cancer Stem Cell Antigen for Adoptive Cell Therapy of Cancer

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There is currently no cure for metastatic, hormone refractory prostate cancer. Localized disease is well controlled with surgery and radiation and in many cases can be curative. However, locally advanced or widespread disease, require hormone therapy for tumor control. This will eventually transform into a hormone refractory state which there are currently no good treatment options. Targeted adoptive immunotherapy against melanoma associated antigens has shown the ability to achieve tumor regression in cases of advanced disease. Prostate stem cell antigen (PSCA) is a well described prostate cancer tumor antigen which can be present on up 48 percent of primary lesions and 64 percent of metastatic lesions (Ross S, et al, *Cancer Res.* May 1, 2002). PSCA expression is not limited to prostate cancer as it is found in up to 60 percent of primary pancreatic tumors (Argani P, et al, *Cancer Res.* June 1, 2001). PSCA is a member of the Thy-1/Ly-6 family of GPI anchored cell surface antigens whose function is unknown. PSCA has low expression in normal prostate tissue and its expression is increased in higher grade and stage tumors making it an ideal target for immunotherapy. Using this model we hypothesize that transduction of T-cells containing a chimeric antigen receptor targeting PSCA can inhibit tumor growth and metastases. We constructed MSGV-1 based retroviral vectors containing six different murine derived single chain variable fragments specific for PSCA linked to T-cell signaling domains CD28 and CD3ζ. The PSCA-CAR's were used to be transduce human peripheral blood lymphocytes with 80 to 90 percent transduction efficiency. Cell lines from melanoma, prostate and pancreatic cancer were screened for PSCA expression via FACS analysis. The PSCA-CAR's were then placed in overnight cocultures with PSCA expressing and PSCA nonexpressing cell lines. Two PSCA-CAR constructs containing single chain variable fragments, m1G8 and bm2B3, revealed a 2 to 3 fold increase in interferon gamma secretion when compared to non-PSCA expressing cell lines. We are in the process of optimizing these constructs and further testing them against PSCA expressing prostate cancer lines. These promising results may provide a new targeted therapy for hormone refractory prostate cancer.

Large-Scale Profiling of Circulating Serum Markers, Single Cell Polyfunctionality and Antigen Diversity of T Cell Response Against Melanoma

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Background: Highly multiplexed, sensitive and single cell profiling at multiple levels is likely to provide important information to advance immunotherapy for cancer, due to complexity of antigen specificity within cancer-targeting T cell populations and large varieties of effector molecules produced. Herein we report on immune monitoring studies using newly developed platforms analyzing samples from 8 patients enrolled in an ongoing clinical trial involving adoptive cell transfer (ACT) of lymphoyctes genetically modified to express a T cell receptor (TCR) specific for MART-1, which are administered to patients with metastatic melanoma together with dendritic cell vaccination after non-myeloablative lymphodepleting chemotherapy.

Methods: Miniaturized, highly multiplexed microchip based technology was adapted to follow the time course of immune responses focused on: (1) melanoma specific T cell repertoire enumeration that simultaneously detected 35 mutated, over-expressed or cancer-germline melanoma antigen specific T cell populations; (2) single cell secretome profiling of 20 cytolytic, inflammatory cytokines and chemokines from sorted phenotypically defined antigen-specific cytotoxic T lymphocytes and different helper T cell subclasses; (3) measurement of 35 blood melanoma tumor markers and immune proteins.

Results: MART-1-specific T cells peaked at 8 to 14 days after ACT with frequencies varying from 1 to 60% of CD3+ T cells in peripheral blood. Over 5 fold expansions in frequency was demonstrated by T cells specific for the melanoma antigens tyrosinase, NY-Eso, Mage 3, Mage 10 and GnT-V ($P < 0.001$), indicating the broadening of immune response. Recovered MART-1-specific T cells had remarkable functional diversity (> 50 different functional subtypes) and polyfunctionality, characterized by strong perforin, TNFα, IL1β and chemokine secretion. However, more than 90% of MART-1 T cells at day 30 lacked TNFα or IFNγ responses, suggesting a dysfunctional or partially exhausted phenotype. Tumor markers and T cell effector proteins in serum, including perforin, granzyme B and cytokines, were significantly released into peripheral blood soon after T cell reinfusion and melanoma associated markers and T cell growth factors followed similar dynamics, which indicated concurrence of tumor destruction and T cell expansion and attack.

Conclusions: Our results indicate importance of epitope spreading, T cell diversity and polyfunctionality for patient's response after the ACT of TCR transgenic lymphocytes; Tumor elimination is closely related to immune response quality. Ongoing experiments will explore quantitatively significance of each marker measured.

Adoptively Transferred Anti-Tumor Th17 Cells are Long-Lived and Evolve Into a Highly Active Memory Population with a Unique Molecular Signature

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Tumor-specific CD4+ T cells polarized under "Th17" conditions efficiently eradicate advanced cancer in mouse models. Th17 cells have been recently described as a meta-stable, short-lived, population with end-effector characteristics and purportedly have a limited ability to form memory (Pepper, *Nature Immunol*, 2010). Although we confirmed that Th17-polarized cells expressed low levels of CD27 compared to Th1-polarized cells, we found that they efficiently survived and formed memory in vivo. While previous work used IL-17 to trace cells after adoptive transfer, in a congenic system we observed that most cells lost the capacity to

produce IL-17A during in vivo expansion. Even though inability of T cells to form memory had been previously attributed to the early loss of CD27 expression we demonstrated that in vitro generated Th17-polarized cells exhibited lower levels of multiple markers of T cell senescence, including Klrg1, FasL, CD25 and PD-1. They also produced more IL-2, had high levels of the pro-survival factors *bcl-2* and *bcl-6* and low expression of its target, *prdm1* that encodes transcriptional repressor Blimp1, a hallmark molecule of terminally differentiated effectors. Using serial global gene expression profiling of adoptively transferred cells, we found that Th17-polarized cells rapidly acquired some Th1-like properties including *t-bet* and *IFN-γ* expression, however Th17-derived memory population was readily distinguished based on its molecular signature and had characteristics closely mimicking a pattern found in highly active CD8+ T memory stem cells described in our previous work. Th17-dervied memory cells expressed high levels of multiple self-renewal and pro-survival-associated transcription factors and regulators, had reduced expression of *prdm1*, as well as low levels of the phenotypic markers of terminal differentiation, including *pd1, klrg1, klrd1* and granzymes, in contrast to higher levels of *CCR7* and *il-7r*. In summary, we report that Th17-derived cells expressed more "stem-like" characteristics distinct form Th1-derived effectors as evidenced by their molecular signature and dramatically enhanced ability to survive and reject tumor. This suggests that Th17-polarizing conditions trigger a distinctive developmental program representing a promising avenue for the development of potent, new T cell-based immunotherapies of cancer and other diseases.

Artifical MicroRNA Targeting Programmed Death Receptor-1 to Enhance Adoptive Cell Transfer Therapy for Cancer

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Melanoma has the unique characteristic of inducing anti-tumor lymphocytes during tumor growth. Tumor Infiltrating Lymphocytes (TIL) demonstrate the ability to recognize and lyse tumor cells in vitro, and the infusion of large numbers of these lymphocytes have resulted in a 49% to 72% response rate in patients with metastatic melanoma.Yet these naturally occurring anti-tumor lymphocytes can often be anergized in vivo, by the suppressive tumor microenvironment. Further improvement to TIL therapy may be realized by targeting negative signaling pathways in the administered TIL.

Programmed death receptor-1 (PD-1) is an immunoinhibitory receptor that is expressed in CD4 and CD8 positive lymphocytes. Interaction between PD-1 and its ligands PD-L1 and PD-L2 deliver a negative signal to lymphocytes. PD-L1 is expressed in a variety of human tumors including melanomas. It was recently shown that in melanoma, PD-1 expressing TIL displayed impaired effector function compared with PD-1 negative TIL. We observed that PD-1 levels were upregulated in TIL and PBL when cocultured with matched melanoma lines in vitro. This suggests that the tumor microenvironment may play a role in the induction and maintenance of PD-1 expression on tumor reactive cells and that PD-1 on TIL may impair the antitumor immune response in patients.

To investigate the PD-1/PD-L1 interaction in a defined in vitro setting, PBL were retrovirally engineered to express PD-1 with either a Mart-1 specific TCR or a HMW (High molecular weight) specific Chimeric Antigen Receptor (CAR). In coculture assays against melanoma lines 1300, 624, 526 engineered to express PD-L1, these Mart-1 TCR/PD-1+ PBL demonstrated impaired tumor recognition with an approximately 50 percent reduction in Interferon-γ (IFN-γ) secretion in the PBL of three donors. HMW CAR/PD-1+ PBL also demonstrated approximately 50 percent reduction in IFN production in a coculture assay with mel 1300, 888, 526, 624 expressing PD-L1.

Antibodies that block the PD-1/PD-L1 interaction are currently in Phase I/II clinical trials, but a more selective approach may be the specific targeting of PD-1 in tumor specific T cells using RNA interference. We are investigating if PD-1 expression in TIL can be silenced using a microRNA-based system. Short hairpin RNA sequences targeting PD-1 were identified to downregulate the expression of PD-1 by 50% to 70% in both T cell lines and peripheral blood lymphocytes. Using these shRNA sequences we are constructing an optimized microRNA-based retroviral vector for the engineering of tumor infiltrating lymphocytes. The downregulation of PD-1 in TIL, may lead to improved anti-tumor activity in vitro and in vivo.

Adoptive T Cell Therapy for Metastatic Melanoma: The MD Anderson Experience

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Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) is a promising treatment for metastatic melanoma. Here, we report on the results of an ongoing Phase II clinical trial testing ACT in metastatic melanoma patients regardless of HLA subtype. Autologous TIL were expanded in large-scale using anti-CD3 and IL-2 and then infused into patients following transient lymphodepletion. This was followed by high-dose IL-2 therapy. The best overall response was determined and correlated with T cell phenotype as well as telomere length. The persistence of specific TCR clonotypes after infusion was also tracked. Altogether, 30 patients have been treated with clinical response data available from 25 patients (as of June 20, 2010). Overall, 13/25 (52%) patients have had a clinical response (PR/CR), with one patient having an ongoing PR for >22 months and another patient having a CR. A higher percentage and number of CD8+ T cells ($P < 0.05$) and a lower percentage of CD4+ T cells ($P < 0.05$) in the infused TIL was associated with a higher probability of clinical response. The degree of tumor shrinkage of major recorded lesions also had a significant correlation with increased percentage and total number of CD8+ TIL infused ($P < 0.05$). Phenotypic analysis using flow cytometry revealed that infused TIL of clinical responders had significantly more CD8+ T cells with a differentiated effector phenotype (CD45RA-CD27-). Unexpectedly, we found that responders had a higher percentage of CD8+ TIL expressing the negative costimulation molecule "B and T lymphocyte attenuator" (BTLA) and were infused with significantly higher numbers of CD8+BTLA+ T cells than non-responders ($P < 0.002$). Tumor regression was also associated with the persistence of dominant TIL TCR V-beta clonotypes in vivo for at least 3 months, while expansion of subdominant TIL clonotypes after 6 months was associated with clinical response after a prolonged period of stable disease in some patients. Interestingly, so far no significant difference in relative telomere length of TIL between responders versus non-responders was evident. In conclusion, this ongoing ACT trial is achieving a high clinical response rate for metastatic melanoma. CD8+ T cells appear to be critical in driving tumor regression. Our results also suggest that the differentiation status of TIL and specific phenotypic subsets (activated/differentiated BTLA+ T cells) are more predictive of clinical response than relative telomere length. In addition, the finding that some patients have delayed clinical responses after a period of stable disease suggests that it is critical not to perform additional therapies on patients compromising T cell function or survival until clear evidence of disease progression is found.

Noninvasive Positron Emission Tomography (PET) Imaging of Sleeping Beauty (SB) Modified CD19-Specific T Cells Expressing Herpes Simplex Virus1-Thymidine Kinase (HSV1-tk)

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PET imaging is an attractive approach to monitor infused genetically modified T cells as it is non-invasive and generates quantitative, longitudinal, and spatial in vivo information about

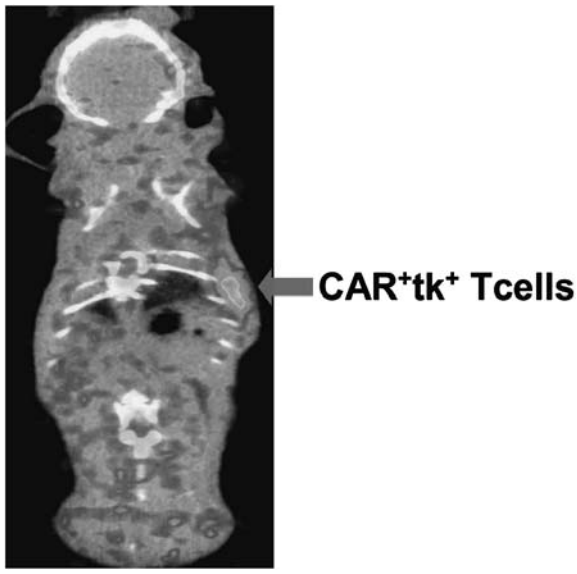

FIGURE 1. Accumulation of ^{18}[F]-FEAU in CAR+tk+ T cells. Mice were anesthesized and CAR+tk+ T cells were injected subcutaneously in the right flank. Positron emission tomography computed tomography (PET/CT) images were acquired 2 hours after intravenous administration of ^{18}[F]-FEAU using Inveon micro-PET/CT scanner. Images were reconstructed by 2-dimensional ordered subsets expectation maximization algorithm. PET and CT image fusion and analysis were performed using vendor software Inveon Research Workplace.

the dynamic status of infused T cells. In this study, we constructed SB DNA transposon vectors encoding a panel of transgenes expressing the wild type HSV1-tk fused to hygromycin phosphotransferase (in vitro selection) and FLAG tag (expression level). Primary T cells were co-electroporated with tkHy SB transposon and a CD19-specific chimeric antigen receptor (CAR) SB transposon and propagated on CD19-specific artificial antigen presenting cells in the presence of cytocidal concentrations of hygromycin B. After 4 weeks of numeric expansion (i) 90% of the T cells were CAR + tk +, (ii) accumulated high amounts of [3H] 2′-fluoro-2′deoxy-1-β-D-arabionofuranosyl-5-ethyl-uracil (iii) were ablated in the presence of ganciclovir and (iv) exhibited redirected killing of CD19 + tumor targets. The CAR + tk + T cells could be visualized by μPET imaging in mice (Fig. 1). This is the first report showing that SB transposition can generate CAR + tk + T cells which can be imaged by μPET in vivo. We have adapted SB system for human application (IND # 14193), and thus this study has immediate translational application to infuse CD19-specific T cells co-expressing HSV1-tk for imaging.

Expansion of Autologous Human Vγ9Vδ2 T Cells Ex Vivo: Potential for Adoptive T Cell Therapy of Prostate Cancer

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Dysregulated cells including solid tumours express stress molecules that can be recognised by cell surface receptors on γδ T cells, including the Vγ9Vδ2 T cell receptor (which recognises pyrophosphate molecules such as IPP that may be expressed on tumour cell surfaces) and the killer activatory receptor NKG2D. Because γδ T cells exhibit marked functional pleiotropy upon activation, we employed extensive microarrays to demonstrate that the use of low molecular weight pyrophosphate antigens in combination with low-dose IL-2 could induce the expression of cytokines and cytolytic agents (eg, Granzymes, Perforin and TRAIL) that are known to be efficacious against tumours. Consequently, in a Phase I trial in which two cohorts of nine stage IV hormone-refractory prostate cancer patients received the amino-bisphosphonate zoledronate (which blocks IPP metabolism) with or without IL-2, we observed profound activation of γδ effector responses in vivo, as well as an indication of therapeutic efficacy in the cohort receiving zoledronate with IL-2.

However, repeated administration of γδ-activating agents can lead to cell exhaustion in vivo; an indication of this is provided by the failure of the bisphosphonate-induced acute phase response to recur after the initial dose. Moreover, a combination of the tumour milieu and concurrent therapies could limit the capacity of some individuals to generate an adequate population of activated γδ T cells able to target the tumour. It is therefore logical to complement bisphosphonate treatment by the activation of autologous γδ T cells *ex vivo* and the subsequent infusion of them. This approach has been validated in mouse models of prostate cancer, and more recently in nude mice bearing human glioblastoma tumours that were treated by adoptive transfer of expanded human γδ T cells. By extension, we are aiming to generate reproducible protocols for the *ex vivo* expansion of human Vγ9Vδ2 T cells, with the objective of adapting the system to GMP for intended treatment of patients with solid tumours. This approach is critically dependent upon the optimisation of key factors, such as the use of cytokines that, in addition to low-dose IL-2, might serve to augment survival and effector potential of such cells in long-term cultures. Furthermore, ongoing work also comprises the development of appropriate functional assays, in order that we can begin to predict in vitro the likely success of subsequent cell engraftment in vivo.

Dissection of Therapy-Induced Melanoma-Reactive Cytotoxic T Cell Responses

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There is strong evidence that melanoma-reactive T cell responses induced by immunotherapeutic interventions such as anti-CTLA4 treatment or TIL therapy can exert clinically meaningful effects. However, at present we do not know which cytotoxic T cell reactivities mediate cancer regression. Furthermore, as the number of 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 more targeted strategies that selectively aim to induce immune reactivity against these antigens.

In the past years 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, and we have now used this platform to monitor immune reactivity against a panel of over 200 melanoma-associated epitopes. First data on the composition and engraftment of TIL products used for adoptive cellular therapy will be presented.

A Data Mining Architecture for Studying Cytokine Production and T Cell Repertoire of Tumor-Specific T Cells Generated from Tumor-Infiltrating Lymphocytes

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Generating T cells with specific reactivity against tumor-associated antigens is a key component of adoptive immunotherapy. In our work, we have established lymphocyte cultures from tumor-infiltrating lymphocytes. After digestion, cells from a specific tumor are grown under different culture conditions such as IL-2, anti-OX40 mAb, NOS inhibitor + Arginase inhibitor, NOS inhibitor + Arginase inhibitor + OX40, IL-15 + Low Dose IL-2, or IL-15 + High Dose IL-2. We refer to the mixed cell population from each of these tumor-culture conditions as CLOIDs. If a particular CLOID was successfully expanded over a 3 to 8 week period, that CLOID was in turn aliquoted and subjected to multiple stimulation conditions such as activation by autologous tumor, allogenic tumor, or anti-CD3. Each of these aliquots were inspected by ELISA for the release of cytokines such as IFN-γ, IL-5, and IL-17. Additionally, the T cell repertoire of each CLOID was examined by multiparameter flow cytometry for subphenotypes as defined by CD4, CD8, CD107a, CCR7, CD45RA, CD27 and CD28 mAb staining. Making sense of this complex data for multiple tumors, multiple culture conditions and multiple stimulation conditions is the challenge addressed here. We created a Rich Analytical Environment by integrating both the ELISA data and the flow cytometry data in a relational database. We also included relevant descriptive data such as source tumor and culture conditions. This approach gave us fast and reliable access to all data from the study. Then, we addressed relevant questions using a combination of visual and statistical techniques. We were able to show that:

For 8 of 9 tumors, there were no statistically significant differences between the number of cells produced by tumor reactive CLOIDs (n = 156 total) digested with enzyme and those digested mechanically.

For some tumors, CLOIDs digested with enzyme produced significantly more IFN-γ than those digested mechanically.

In tumors digested with enzyme, the autologous tumor-specific central memory (CCR7 + CD45RA − CD27 + CD28 +) and central memory-like (CCR7 + CD27 + CD28 +) CD4 and CD8 T cell populations were significantly higher when cultured with IL-15 and low dose IL-2 than with high dose IL-2 alone ($0.001 < P < 0.007$).

In conclusion, supporting this complex experimental design with a sound data architecture enabled us to identify a variety of important findings.

Improved Persistence and In Vivo Antitumor Efficacy of Human T Cells Engineered to Express a 4-1BB Costimulated Alpha-Folate Receptor-Specific Chimeric Immune Receptor

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Background: α-Folate receptor (αFR) is a folate-binding protein overexpressed on 90% of ovarian cancers. Here we constructed αFR-specific single-chain fragment (scFv)-based chimeric immune receptors (CIRs) with or without novel costimulatory signaling motifs and evaluated the antitumor function of CIR transduced human T cells against human ovarian cancer cells in vitro and in vivo.

Methods: Novel CIRs were constructed that contain a αFR-specific scFv (MOv19) coupled to either an inactive form of the CD3-ζ intracellular domain (MOv19-Δζ), the CD3ζ chain signaling module alone (MOv19-ζ), or in combination with the CD137 (4-1BB) costimulatory motif (MOv19-BB-ζ). Human T cells were transduced with recombinant lentiviral vectors for CIR expression. In coculture assays, CIR transduced T cells were measured for reactivity against ovarian cancer cells expressing αFR via IFN-γ ELISA and cytokine bead assay. Cytotoxicity was measured using a bioluminescence system in vitro and in a xenogeneic model of human ovarian cancer in vivo.

Results: Primary human T cells were engineered to express scFv Mov19 via lentiviral transduction with a 50% transduction efficiency. Using in vitro assay systems, both MOv19-ζ and MOv19-BB-ζ transduced T cells demonstrated target-specific release of IFN-g, TNF-α and IL-2 cytokines and cytotoxic function when cocultured with αFR + tumor cells, while T cells transduced with MOv19-Δζ or with GFP did not. However, in immunodeficient NSG mice bearing large, established s.c. human ovarian cancer (SK-OV-3), only transfer of human T cells engineered to express the costimulated MOv19-BB-ζ CIR mediated tumor regression. The anti-tumor response was achievable by i.v., i.p. or i.t. T cell infusion and was associated with the selective survival of human T cells only in mice receiving costimulated MOv19-BB-ζ CIR. In contrast, T cells transduced with MOv19-ζ, MOv19-Δζ or gfp did not persist and had little to no effect on tumor outgrowth.

Conclusions: Our results demonstrate direct and specific tumor recognition and killing of αFR + ovarian cancers cells by T cells genetically engineered to express MOv19 CIRs in vitro and in vivo and show that incorporation of the 4-1BB (CD137) signaling domain into CIRs can enhance T cell survival and antitumor activity in vivo.

Enrichment of AML-Specific T Cells with Superior Functional Capacity and Transgenic Receptor Expression for Adoptive T Cell Transfer

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The majority of patients with acute myeloid leukemia (AML) reach a complete hematological remission after intensive chemotherapy. However, only 20% to 40% of all patients with AML achieve a disease-free survival of more than 5 years. Therefore, long lasting therapy approaches are urgently needed. T-cell-based immunotherapy transfering high avidity AML-specific CD4 + and CD8 + T cells constitutes a new encouraging therapeutic strategy.

In order to obtain high avidity T cell receptors (TCR) directed against multiple epitopes specific for AML-associated antigens, we established an allogeneic priming approach utilizing matured dendritic cells (mDC) that have been electroporated with in vitro transcribed (ivt) RNA encoding a tumor-associated antigen and HLA-A2. Furthermore, optimization of monocyte-derived mDC prepared within 3 d with excellent capacity to prime efficient T cell responses was achieved by including Toll-like receptor agonists into the DC maturation cocktail. Recent studies revealed that upregulation of CD137 after TCR triggering can be used to select antigen-specific T cells without reliance on MHC-multimers. Therefore, we performed a mulitimer-independent CD137-positive enrichment step after specific restimulation of AML-specific T cell lines. Clones were developed from enriched T cells.

By this technology, using the hyaluronan-mediated motility receptor (HMMR) as an AML-associated antigen, we were able to achieve ten-fold more T cell clones, compared to autologous priming approaches. Additionally, we increased the yield of AML-specific clones by means of the CD137-based enrichment step from 11% to 52%. Furthermore, using this priming approach, we were able to obtain 15 T cell clones, which showed a specific lysis of greater than 50%, and 60% of those clones secreted high amounts of IL-2, IFN-γ, MIP-1α and TNF-α, a feature of polyfunctionality. Employing retroviral transduction technology, we could transfer the functional capacities of induced HMMR-specific TCR into recipient PBL, overcoming the need for extensive culture of the original clones.

Current experiments are focusing on functional comparisons between original T cell clones and tg-TCR PBL in vitro and in vivo. Moreover, we will analyze TCR with various functional avidities for cross-reactive recognition of self-peptides in an in vivo model system as well as in vitro.

Fratricide of Recipient Lymphocytes Expressing Surviving-Specific Transgenic T Cell Receptors

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Expression of T cell receptors (TCR) as transgenic proteins in peripheral blood lymphocytes (PBL) enables T cells with defined tumor specificity to be generated in high numbers for patient-individualized therapy (TCR gene therapy). Hereby, the selection of tumor-associated antigens (TAA) that can be effectively targeted on tumor cells is of central importance. Survivin, a well-characterized inhibitor of apoptosis, has been suggested as a canditate TAA because of its potential role in oncogenicity and its broad expression in most tumors but absence from most normal adult tissues. To explore use of survivin as a target antigen for TCR gene therapy, we generated HLA-A2 allo-restricted survivin-specific T cells with high functional avidity.

We isolated several high affinity TCR recognizing peptides derived from survivin presented by HLA-A2 molecules. Following transfer of these TCR into HLA-A2-recipient lymphocytes, we found that the effector cells displayed excellent specific killing of HLA-A2+survivin+ tumor cells, indicating their therapeutic potential. Surprisingly, when these TCR were expressed as transgenic proteins in HLA-A2+ recipient lymphocytes, as they would be applied in the clinical setting, we discovered that the recipient lymphocytes underwent extensive apoptosis over time. This demise was caused by HLA-A2-restricted fratricide that occurred due to survivin expression in recipient lymphocytes, which created ligands for transgenic TCR recognition. Furthermore, we could demonstrate that cytotoxic T cell clones of various specificities derived from HLA-A2+ donors were directly killed by survivin-specific TCR-modified PBL.

Therefore, survivin-specific TCR gene therapy would be limited to application in HLA-A2-mismatched stem cell transplantation. However, these results raise a general question regarding cancer vaccines targeting proteins that are also expressed in activated lymphocytes, since induction of high avidity T cells that expand in lymph nodes following vaccination or later accumulate at tumor sites might limit themselves by self-MHC-restricted fratricide and also eliminate neighboring T cells of other specificities.

Therapeutic Cell Engineering Using Surface-Conjugated Synthetic Nanoparticles

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Adoptive cell therapy (ACT) with tumor-specific T cells is a promising approach for cancer therapy, but strategies to enhance the persistence and functionality of ACT T cells are still sought. Meanwhile, the use of synthetic nanoparticles as carriers to deliver drugs to tumor environments has become of increasing interest, with the goal of targeting drugs to tumor sites. We will describe a strategy combining these two approaches, based on the chemical conjugation of adjuvant drug loaded nanoparticles (NPs) to lymphocytes for ACT. Using a simple ex vivo conjugation process, drug-loaded particles are attached to T cells without interfering with intrinsic cell functions, including tumor/lymphoid tissue homing. We demonstrate how ACT T cells carrying cytokine-loaded NPs (to permit pseudo-autocrine self-stimulation following transfer into tumor-bearing hosts) are capable of massive in vivo expansion and robust anti-tumor responses, enabled by minimal doses of cytokines that by comparison have no therapeutic effect when given in a soluble form systemically. This approach is a facile and generalizable strategy to augment cytoreagents while minimizing systemic side effects of adjuvant drugs. Based on the wealth of available NP-formulations tailored to deliver small molecule drugs, proteins, or siRNA, the range of therapeutic or diagnostic cargos that can be attached to therapeutic cells extends far beyond the small molecules and recombinant proteins tested in our studies. In addition, our results suggest therapeutic cells are promising vectors for actively targeted drug delivery.

Development of a Novel Chimeric Antigen Receptor Targeting the Melanoma Antigen GP75

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Adoptive transfer of tumor-specific T cells is an effective method for treating established tumors. Unfortunately, tumor-specific T cells in cancer patients are typically low in abundance and require extensive culture ex vivo to produce cell numbers that are suitable for infusion. This limitation can be overcome by engineering T cells to express chimeric antigen receptors (CARs) where the intracellular signaling components of the T cell receptor are fused to an extracellular binding domain with specificity for a target on the tumor cell; a single chain antibody is typically used to target the CAR. These CARs can be delivered into large populations of naïve T cells, facilitating both the production of tumor-specific T cells and polarization towards specific phenotypes. We have developed a novel CAR directed against the melanoma antigen GP75 by linking a single-chain antibody derived from the TA99 hybridoma to a synthetic receptor scaffold comprised of the CD8 hinge (or IgG1 hinge for greater flexibility), the CD28 transmembrane and signaling domains, and the signaling domain from CD3ζ. Recombinant MSCV expressing the GP75-CARs were prepared and used to transduce T cells. The retroviruses transduced murine T cells with high efficiency (about 60%) and we are currently testing their functionality in vitro and in vivo. Although GP75 is present on the tumor cell membrane in vivo, surface expression is weak in vitro. Therefore, we have also generated target lines that express a truncated GP75 protein which is localized to the surface of the tumor cell for in vitro studies. The results of our optimization studies and in vivo experimentation will be discussed. These CARs represent important tools for developing new methods employing CAR-engineered T cells in an immune competent mouse model.

Generating Potent Anti-Tumor CD62Lhigh CD8+ T Cells for Adoptive Immunotherapy

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The T lymphocyte pool can be sub-divided into a naïve population (Tn) of antigen inexperienced cells, effector memory (Tem), and central memory (Tcm) T cells. The memory T cells mount a fast and potent recall immune response following antigen recognition and Tcm cells may contain long-term self-renewal T memory cells. Animal studies suggest that Tcm cells not only survive longer after adoptive cell therapy, but also confer superior anti-tumor reactivity compared to Tem cells, including eradication of large established tumors. Based on the differentiation markers L-selectin/CD62L and CD45RO, and the release of the cytokine IL-2, Tcm can be distinguished from Tem cells. Antigen-specific CD8+ T cells can be used to treat cancer and prevent infections in humans. The genetic modification of CD8+ T cells using anti-tumor T cell receptors is a promising approach for the adoptive cell therapy of patients with cancer. We developed a simplified method for the

clinical-scale generation of Tcm CD8+ T cells following transduction with lentivirus encoding anti-tumor T cell receptors. Briefly, the isolated CD8+ T cells were activated using plate-bound OKT3 overnight followed by lentiviral vector mediated transduction. Six hours later, the irradiated feeder cells from a pool of three allogeneic PBMC were added in a ratio of feeders to CD8+ T cells (10:1) in the presence of 30 ng/mL OKT3 and maintained for 12 days in vitro culture. The TCR engineered CD8+ T cells conferred specific anti-tumor activity and the majority displayed a central memory-like phenotype. In this study, we optimized the culture conditions of in vitro generated anti-tumor CD8+ T cells towards the CD62Lhigh phenotype. We compared different cytokines or combinations of IL-2, IL-7, IL-12, IL-15 and IL-21 to expand genetically engineered CD8+ T cells. IL-7, IL-21 or their combination could increase the percentage of CD62L+ cells compared to IL-2. IL-12 alone yielded the highest percentage of CD62L+ cells (up to 97%), when CD62LhighCD8+ T cells polarized by IL-12 were co-cultured with tumor lines, this population secreted the high levels of IFNγ and IL-2. The methodology that we developed for generating anti-tumor CD62Lhigh CD8+ T cells ex vivo may be ideal for the adoptive immunotherapy of cancer in humans.

Improving Adoptive Cell Therapy by Blocking the TGFβ Pathway at the Tumor Environment

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The role of TGFβ in cancer biology is complex and may involve tumor promotion or tumor suppression, depending on when and where this cytokine is expressed. It has been reported that multiple types of tumors can secrete TGFβ at the tumor site and may, through induction of T regulatory cell (Treg) generation and inhibition of CTL and NK cell cytolytic activity, evade immune surveillance. In our lab, we have demonstrated that exogenous TGFβ1 attenuated the effector function of antigen specific T cells. In an effort to modulate TGFβ pathway in the tumor environment, we engineered antigen specific T-cells with a γ-retroviral vector encoding soluble forms of TGFβ receptor II. The genetically modified T cells would traffic and secret soluble receptor at the tumor site. We constructed two vectors expressing different forms of soluble receptor, one soluble TGFβ receptor II (sTGFβRII) and the other soluble receptor fused with human Fc IgG (sTGFβRII Fc). In vitro data indicated that transduced PBLs secreted soluble receptors, which could be detected by western blot. The PBLs secreting soluble receptors also displayed reducing sensitivity to exogenous TGFβ1 treatment compared with control cells, as measured by decreased phosphorylated smad2 protein detected by western blot. Currently, we are investigating the therapeutic efficacy of this strategy in vitro and eventually in vivo.

Protein L: A Novel Reagent for Universal Detection of Chimeric Antigen Receptor (CAR) Expression by Flow Cytometry

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There has been significant progress in the last two decades on the design of chimeric antigen receptors (CAR) for adoptive tumor immunotherapy targeting a variety of tumor-associated antigens. CARs consists of a single chain antibody fragment directed against a tumor-associated antigen fused to an extracellular spacer, transmembrane domain, and T cell cytoplasmic signaling moieties such as CD3 zeta, CD28, or 4-1BB. Currently several clinical trials are underway using gene modified peripheral blood lymphocytes (PBL) with CARs directed against a variety of tumor antigens (eg, ERBB2, CEA, CD19, CD20, and PSMA). Despite the improvements in the design of CARs and expansion of the number of target antigens, there is no universal flow cytometric method available to detect the expression of CARs on the surface of lymphocytes. To determine the level of expression of CARs on gene-modified lymphocytes by flow cytometry, most investigators use anti-Fab conjugates but these reagents are generally polyclonal antibody fragments and are species specific. To address this issue for the detection of any antibody-based CAR, we employed protein L as a universal reagent to determine the expression of various types of CARs on transduced lymphocytes. Protein L is an immunoglobulin-binding protein that binds to the variable light chains (kappa chain) of immunoglobulins without interfering with antigen binding site. Protein L binds to all classes of Ig (IgG, IgM, IgA, IgE and IgD), and also binds to single-chain antibody fragments (scFv) and Fab fragments. To evaluate the suitability of protein L as a reagent to determine the cell surface expression of CARs; activated human PBLs were retrovirally transduced with five different CARs expression vectors. CARs used were based on both human antibodies (anti-EGFRvIII, anti-ERBB2, and anti-VEGFR2), and murine derived antibodies (anti-HMW-MAA, and anti-CD19). Transduced cells were stained with Biotin labeled Protein L (GeneScript, cat. number M00097) at 100 ng per $1 \times 10e6$ cells followed by incubation with PE-conjugated streptavidin and analysis by flow cytometry. For comparison, cells were stained in parallel with biotin conjugated goat-anti-mouse or anti-human Fab antibodies. Using Protein L, all CAR transduced lymphocytes exhibited specific staining pattern (compared to untransduced cells) and staining was more uniform than using the anti-Fab antibodies. Our data demonstrate the feasibility of employing Protein L as a universal reagent for the detection of CAR expression on transduced cells by flow cytometry.

CLINICAL TRIAL ENDPOINTS

The Impact of Storage Temperature on PBMC Isolation, Viability and Immunologic Function

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Correlative studies of immune function in multicenter trials of immune therapy depend on preservation of lymphocyte viability and function. Identifying factors that compromise specimen integrity during shipment will lead to measures to eliminate those variables. The effect of temperature during the storage of peripheral blood and the subsequent processing, recovery and function of the lymphocytes was studied in two ways. First, whole blood specimens from patients on two vaccine-based clinical trials were monitored over a 9-month period for high and low ambient temperatures encountered during overnight shipment from off-site trial centers prior to Ficoll gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC) and cryopreservation. Effects of ambient temperature during shipment on cell yield, recovery and viability, were evaluated. In addition, heparinized blood from healthy donors was collected and stored at temperatures of 15°C, 22°C, 30°C and 40°C for varying lengths of time before isolation of PBMC in order to simulate blood collection and shipping practices of whole blood in various ambient temperatures/weather conditions. Our hypothesis was that extreme temperatures encountered during storage and transport of blood specimens diminishes lymphocyte viability, recovery and function. Measures for specimen integrity included cell yield, recovery from liquid nitrogen storage, viability and immunologic function of the isolated lymphocytes. During shipment, warmer ambient temperatures favored greater cell yields of PBMC following isolation and recovery from cryopreservation. In the simulated storage conditions, exposure of whole blood to temperatures below room temperature (22°C) resulted in a suboptimal yield of PBMC after Ficoll separation ($P = 0.003$ for 12 h at 15°C). When compared to blood stored overnight at room temperature, reduced cell recovery following cryo-preservation as well as decreases in cell viability and immune function were observed in specimens exposed to 15°C ($P = 0.031$) or 40°C ($P = 0.026$) for greater than 8 hours.

Improvement of these parameters was seen in blood specimens stored at 30°C prior to processing for all time points tested. Excellent viability and function were obtained even after 24 hours at 22 to 30°C. Therefore, considerations in the design of shipping containers should be to protect against extreme ambient temperatures and to maintain specimen temperature near 30°C.

Decoding the Tower of Babel

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The past two decades have seen a dramatic increase in the number of phase II and III trials evaluating biologic therapy for the treatment of solid tumors, especially melanoma, yet very few new biologic agents have been approved for widespread use. In fact, in melanoma, no new biologic agents have been approved by the US FDA since high-dose interferon alfa in 1995 and high-dose interleukin-2 in 1998. Even those approvals have not resulted in the uniform adoption of the approved drugs-only a minority of melanoma patients meeting the label indications for these two drugs ever receive them. Worldwide, these two drugs are used even more infrequently than in the US.

Although the reasons behind the limited penetration of biologic therapy into the routine management of melanoma and other solid tumors are manifold, a recurring theme has been the inability to agree on fundamental principles relating to clinical trial design and interpretation, which in turn complicates acceptance of clinical trial results and makes it more difficult to identify the best agents and regimens for testing in large-scale studies. Examples of the contentious issues that have faced melanoma clinical trialists include choosing the optimal choice of "control" regimens against which investigational drugs should be compared, whether placebos should be utilized, and identifying the ideal patient population to study (examples include allowing previously treated vs. treatment naïve patients; restricting eligible populations based on stage, serologic markers of disease burden, HLA haplotype, or the presence of a mutation or expression of defined antigens on a tumor biopsy specimen; and allowing or excluding patients with central nervous system metastasis). But perhaps no area has been more controversial than the optimum choice of endpoints for melanoma clinical trials. Improvement in overall survival is widely accepted as the ultimate proof that a therapy has provided clinical benefit, but is it always required for a biologic therapy to be adopted? A number of factors can limit the ability of a clinical trial to detect an overall survival benefit with the use of an efficacious therapy, such as crossover (either explicitly as part of the study design or more insidiously in cases of patients crossing over to the same or a closely related drug once off-study), salvage therapy with a different class of effective agent, and early termination of a trial due to an evident progression-free survival or objective response advantage at an interim analysis. In the past, the likelihood of salvage therapy resulting in improved survival for patients failing protocol therapy was considered remote, but that can no longer be assumed to be the case. Objective response rates, widely used in other solid tumors particularly to identify which investigational therapies merit testing in phase III trials, have proven to be poor predictors of success for phase III trials of biologic therapy in melanoma. Whether modified criteria tailored to detect "atypical" response patterns associated with some modern forms of biologic therapy remains to be seen, and another relatively unrecognized problem with modified criteria is that we do not have any validated database for how often non-biologic agents, like single or multi-agent chemotherapy, show atypical response patterns in melanoma. It is not safe to assume that atypical response patterns never or rarely occur with melanoma chemotherapy. Regardless, objective response as an endpoint is not useful for agents that act by delaying tumor progression without leading to substantive shrinkage of individual tumors. It is not at all unreasonable to expect many biologic agents to exert a major antiproliferative effect even if they do not cause tumor regression, and for these agents objective response would be a poor endpoint for clinical trials.

Progression-free survival is an endpoint that encompasses both tumor regression and stabilization without regression, and so is an attractive alternative to objective response rate. However, assessments of progression can be subjective-and hence subject to observer bias in unblinded studies. Moreover, some patients with slowly progressing tumors meet criteria for "stable disease," overstating the effect of treatment. But the biggest criticism of progression-free survival as an endpoint is that minor delays in time to progression do not constitute clear evidence of "clinical benefit" of therapy. For all these reasons, progression-free survival benchmarks-most commonly the percentage of patients who are alive and progression-free at 6 months after protocol entry-have become increasingly popular as a relatively objective endpoint with readily apparent clinical benefit. The six-month progression-free survival percentage is often paired with the twelve-month overall survival percentage.

A recent meta-analysis tabulated the six-month progression-free and twelve-month overall survival percentages for 70 phase II trials involving 2100 stage IV melanoma patients and calculated 95% confidence intervals around the median. These confidence intervals, which take into account the size of the trial, serve as another barometer of whether the results seen with a new agent in early phase trials are sufficiently interesting to merit testing in a randomized phase III trial. Like all trial endpoints, they have their limitations, and ongoing clinical trial experience is beginning to bring some of these limitations to light. It remains unknown which of these two benchmarks is most predictive of efficacy, and it is also unclear how these benchmarks will translate to study populations other than the relatively unfavorable cooperative group melanoma patients that comprised the meta-analysis. Nonetheless, using these benchmarks and understanding the meta-analysis results can clearly help us decode the Tower of Babel, and help us recognize and develop active biologic agents in melanoma more quickly and effectively than in the past.

COUNTERING NEGATIVE REGULATION

Immune Performance Profile in Pancreatic Adenocarcinoma Patients

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Pancreatic cancer presents an enormous therapeutical challenge as naturally resists to standard therapies. Among the alternative approaches, immunotherapy seems to be one of the most promising. However, a limitation can be the severe immune dysfunction due to the combinatory effects of several mechanisms presents in pancreatic cancer patients.

Some tumor escape mechanisms are directly associated to the antigen recognition, others instead act on the immune status activation stimulating some suppressive population as T regulatory cells (Treg) or Myeloid Derived Suppressor Cells (MDSC). Others involved an immune dysfunction at the immune mediators receptors signaling pathways level.

The overall effect is a generalized immune suppression, however we want to prove with the present study that these mechanisms are responsible at a different extent in each single cancer patient.

To test the patient specific "immune performance profile" we recruited 30 patients diagnosed with locally advanced or metastatic pancreatic ductal adenocarcinoma and 17 healthy donors. We performed a phenotypical analysis to test the presence of Treg cells (CD4 CD25 Foxp3 positive cells) and MDSC (CD14 and IL4Rα positive cells) in PBMC and of MDSC in PMN (CD15 and IL4Rα positive cells).

We noticed that this phenotypic profile, divided in several subgroups the patients tested. Some have a profile similar to the majority of healthy donors while others are characterized by a different relative frequency of the analyzed immunosuppressive subpopulations. Interestingly we noticed that metastatic patients have a more homogeneous profile according to the presence of immune suppression and those profiles are similar to the one characteristic for healthy donors.

We further demonstrated that CD14 positive cells sorted from PBMC of patients that expressed higher level of IL4Rα suppress in a stronger way both antigen specific and antigen aspecific lymphocytes proliferation.
We added as part of the "immune performance profile": a proliferation assay to assess the responsiveness of lymphocytes to OKT3 plus IL2 stimulation and the speed of phosphorylation of STAT1 after IFNα stimulus. Using these new parameters we were again able to discriminate different subgroups of patients characterised by a different behaviour. In conclusion, our study demonstrated that each patient is characterized by a unique immune performance profile. The understanding of its uniqueness can represent a crucial help for the choice of the suitable customised therapy, in the context of combinatory therapies, often aimed to reinforce the patient immune system before immunotherapeutic intervention.

The Multikinase Inhibitor Sorafenib Reverses the Suppression of IL-12 and Enhancement of IL-10 by PGE2 in Murine Macrophages

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Classical activating stimuli like LPS drive macrophages to secrete a battery of inflammatory cytokines, including interleukin (IL)-12/23, through toll-like receptor (TLR) signaling. TLR activation in the presence of some factors, including prostaglandin E2 (PGE2), promotes an anti-inflammatory cytokine profile, with production of IL-10 and suppression of IL-12/23 secretion. Extracellular signal regulated kinase (ERK) is a key regulator of macrophage IL-10 production. Since it inhibits ERK, we investigated the impact of Sorafenib on the cytokine profile of macrophages. In the presence of PGE2, Sorafenib restored the secretion of IL-12 and suppressed IL-10 production. Moreover, IL-12 secretion was enhanced by Sorafenib under conditions of TLR ligation alone. Furthermore, the impact of tumor culture supernatants, cholera toxin, and cAMP analogs (which suppress IL-12 secretion), was reversed by Sorafenib. Sorafenib inhibited the activation of the MAP-kinase p38 and its downstream target mitogen and stress activated protein kinase (MSK), and partially inhibited protein kinase B (AKT) and its subsequent inactivation of the downstream target glycogen synthase kinase 3-β (GSK-3β). Interference with these pathways, which are pivotal in determining the balance of inflammatory versus anti-inflammatory cytokines, provides a potential mechanism by which Sorafenib can modulate the macrophage cytokine phenotype. These data raise the possibility that the use of Sorafenib as cancer therapy could potentially reverse the immunosuppressive cytokine profile of tumor-associated macrophages, rendering the tumor microenvironment more conducive to an anti-tumor immune response.

Reversal of Immune Supression in Cancer by Manipulation of Tumor Iron Metabolism

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Introduction: Tumor immune escape mechanisms are numerous, extremely complex, and contribute to cancer growth and progression. We investigated the role of iron on natural killer cell (NK) cytotoxicity and the effect of downregulating the iron storage protein heavy chain ferritin (Fer-H) on MCF-7 cell growth. Methods: The role of nitric oxide (NO) mediated NK cell cytolysis of MCF-7 breast cancer cells was evaluated in the presence of increasing concentrations of iron, as well as in the presence of the iron chelator deferoxamine (DFOM). The effect of antisense oligonucleotides targeted to Fer-H in MCF-7 cells was also investigated.
Results: Iron inhibited the NO associated cytolysis of tumor cells by NK cells and protects tumor cells from NK cell mediated immune responses. DFOM increased NK cell cytolysis of MCF-7 breast cancer cells after four hours incubation, but the concentration was critical. Downregulation of Fer-H with a targeted antisense oligonucleotide synergistically increased the antitumor activity of rTNFa against the MCF-7 human breast cancer cell line. The Fer-H inhibited TNFa induced apoptosis by suppressing reactive oxygen species (ROS).
Conclusions: Increased iron in tumor cells and their microenvironment protects the tumor from T cell cytotoxicity. Therefore, manipulation of iron metabolism in tumor cells and their microenvironment may help reverse immune suppression.

Induction of Different CD4+ T Cell Subsets by HLA-DR- Myeloid Derived Suppressor Cells and CD14+ HLA-DR+ Monocytes

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The observation that tumors progress in patients with cancer despite the presence of tumor-specific immune responses suggests that tumor development leads to a number of immune suppressor mechanisms, which are important to consider when designing immunotherapy protocols. These mechanisms include, but are not restricted to production of immunosuppressive cytokines and prostaglandins, impaired antigen-presenting cells, generation of inhibitory macrophages, increase in regulatory T cells and induction of myeloid derived suppressor cells (MDSCs). We have previously identified human CD14+ HLA-DR- MDSC in patients with cancer. These cells suppress directly the function of CD4+ and CD8+ T cells as well as indirectly through the generation of CD4+ regulatory T cells (Hoechst et al *Gastroenterology*. 2008 and Hoechst et al *Hepatology*. 2009). We have now extended our study to investigate what type of CD4+ T cells are induced by the remaining CD14+ cell population in vitro. Methods: Human CD14+ HLA-DR- MDSCs, and CD14+ HLA-DR+ monocytes were isolated from peripheral blood of healthy volunteers and co-incubated with CD3/CD28/CD2 stimulated naïve CD4+ T cells. Naïve CD4+ T cells were analyzed after 4 and 7 days of in vitro culture and analyzed for phenotype and function. Results: In this study, we show that myeloid cells of different origin can impact differentiation of naïve CD4+ T cells in human peripheral blood. We have found that human CD14+HLA-DR- MDSCs induce Foxp3+ regulatory T cells, whereas CD14+HLA-DR+ monocytes lead to generation of IL-17 secreting, RORc+ Th17 cells when co-cultured with naïve autologous CD4+ T cells. Discussion: Based on our data, we propose that the balance between these two subsets of myeloid cells plays a crucial role in immune regulation and can define the outcome of immune response.

Loss of HLA-DR Expression on CD14+ Cells; A Common Marker of Immunosuppression in Cancer Patients

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Cancer-associated suppression of immunity must be countered if immunotherapy is to be effective. To study the nature of this immunosuppression, we have systematically assessed patient immunity by phenotyping leukocytes directly from peripheral blood. We have used this approach to qualitatively and quantitatively measure 40 phenotypes in more than 145 patients representing several diseases including glioblastoma, lymphoma, and prostate cancer. Cancer patients (prior to treatment, or more than 2 mo after treatment) are characteristically lymphopenic, with the deficit primarily due to loss of CD4 T cells, and heterogeneous levels of regulatory T cells. However, the most profound change is

the loss of MHC class II expression (measured via HLA-DR) on CD14+ monocytes. In all malignancies measured to date, CD14+HLA-DRlo/neg monocytes are significantly elevated in peripheral blood of patients including glioblastoma patients (about 24 ± 20.0% of CD14+ monocytes were HLA-DRlo/neg; n = 24), lymphoma (27.8 ± 3.1%; n = 40), and prostate cancer (30.7 ± 15%; n = 22) compared to healthy donors (8.5 ± 8.0; n = 15). These percentages were converted into absolute cell counts and, in healthy donors, the absolute cell count of CD14+HLA-DRlo/neg monocytes ranged between 8 to 70 cells/μL with a median of 35 cells/μL compared to 134 cells/μL (range of 1 to 794 cells/μL) in GBM patients, 155 cells/μL (range of 25 to 591) and for lymphoma patients. These values have a large dynamic range in cancer patients, suggesting clinical and pathological heterogeneity within disease categories. CD14+HLA-DRlo/neg monocytes are functionally immunosuppressive as they inhibited T cell proliferation in an antigen independent manner. Most significantly, CD14+HLA-DRlo/neg monocytes were refractory in their ability to differentiate into mature dendritic cells that was independent of the maturation signal used. These findings have significant implications on the generation (both in vitro and in vivo) of DC for clinical use. Independently, others have shown CD14+HLA-DRlo/neg monocytes to be prognostic in the outcome of other immunosuppressive settings like sepsis. Our immunophenotyping analysis of a cohort of patients with/or at risk of acute lung injury with or without infection revealed that these patients had a median of 264 cells/μL (range of 31 to 1443; n = 29). Our data suggests that CD14+HLA-DRlo/neg cells may be a useful marker of immunosuppression in cancer patients as well as for patients at risk of infection. As such, the immunosuppressive functions of CD14+HLA-DRlo/neg monocytes should be considered during the development of novel biological therapies of cancer.

Blockade of the PD-1 Pathway Improves Immunotherapy for Multiple Myeloma

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Multiple Myeloma is an incurable plasma cell malignancy. Patients who fail conventional therapy are treated with high-dose therapy and hematopoietic stem cell transplantation (HSCT). This treatment typically reduces the tumor burden and can induce remission, but these patients frequently relapse from sites of chemotherapy-resistant disease. We developed a combined immunotherapy approach that incorporates HSCT, adoptive transfer of T lymphocytes, and administration of a cell-based vaccine. This approach has been effective in a murine neuroblastoma model, but it confers no significant improvement in survival in mice with established myeloma (5T33 tumor line). Therefore, we sought to identify the mechanisms of immune evasion utilized by this myeloma. We observed high expression of B7-H1, the ligand for the inhibitory receptor PD-1, on 5T33 tumor cells. We also observed increased expression of PD-1 on both CD4 and CD8 T cells in tumor-bearing mice, and increased frequencies of PD-1-expressing T cells were found only in lymphoid tissues containing tumor cells. Interestingly, we observed a similar pattern of expression when we evaluated clinical samples from multiple myeloma patients. We found increased B7-H1 expression on plasma cells in marrow samples from myeloma patients. Furthermore, we found that patient T cells displayed increased levels of PD-1. In our murine model, the increased expression of PD-1 on T cells correlated with tumor burden and was restricted to areas of local tumor accumulation. When PD-1 or B7-H1 blockade was used together with combined immunotherapy (HSCT, adoptive T cell transfer and vaccination) in tumor-bearing mice, we observed a significant improvement in survival (40% survival in treated mice vs. 0% survival in control mice). These data demonstrate an important role for the PD-1 pathway in suppressing immune responses to myeloma and suggest that blockade of this pathway may enhance immunotherapy for this disease.

TLR Ligands Induce SOCS2 Gene Expression in Human Monocyte-Derived Dendritic cell via NF-κB

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The suppressor of cytokine signaling 2 (SOCS2) negatively regulates cytokine signaling, and STAT5b has been demonstrated as its regulatory transcription factor upon GH signaling. We previously reported that SOCS2 is induced by LPS stimulation in human DCs. Here we further investigate the transcriptional regulation of SOCS2 expression.

Methods: Human DCs Generation: iDCs was generated by culturing monocytes with 50 ng/mL GM-CSF and 20 ng/mL IL-4 for 6 days. RT-PCR: Total RNA was extracted using TRIzol and reverse-transcribed for real-time PCR analysis. Bioinformatics: Genomic sequence of human SOCS2: UCSC. Prediction of transcription factor binding site (*http://www.genomatix.de*). Western blot: The cell lysates were submitted to SDS-PAGE, transferred to PVDF membranes for Western Blot analysis.

Result: TLR ligands induce SOCS2 expression. iDCs were treated with various TLR ligands including Pam3CSK4(TLR1/2, 10 μg/mL), LPS(TLR4, 200 ng/mL), flagellin(TLR5, 1 μg/mL), poly-I:C(TLR3, 30 μg/mL), imiquimod(TLR7, 5 μg/mL), ssRNA40 (TLR8, 5 μg/mL) and CPG-ODN2336(TLR9, 5 μg/mL) for mRNA analysis. Real-time PCR result revealed that SOCS2 mRNA was induced by TLR 1/2, TLR 4, TLR 5, TLR 8 and TLR 9 signaling from 8 hours till 24 hours. No SOCS2 increase on TLR 3 and 7 stimulations. TLR 1/2 and 4 stimulation induced the strongest SOCS2 induction. The effect was moderate on TLR5, TLR8 and 9. NF-κB was predicted as transcription factors for human SOCS2 gene. STAT 3, 5 and 6, NF-κB, IRF 1, 3, 4 and 7 were found as putative SOCS2 transcriptional factors in human SOCS2 promoter region for TLR4 signaling.

NF-κB is a transcription factor for human SOCS2 expression in TLR4 signaling.

After inhibiting NF-κB pathway by pre-incubating iDCs 1 h with BAY11-7082 (10 μM), a IκBa phosphorylation inhibitor; BMS-345541 (20 μM), a allosteric site-binding IKK-2 inhibitor, proteasome inhibitor MG-132 (20 μM) and protein synthesis inhibitor cyclohexamide (20 μg/mL), we stimulated the cells with LPS and extract whole protein for western blot analysis. The SOCS2 protein became significant after 8 hours and strong after 24 hours stimulation. The various NF-κB inhibitors inhibited SOCS2 expression demonstrating that NF-κB was necessary for the induction of SOCS2 transcription on TLR4 signaling.

Discussion: SOCS2 expression is different after various TLR ligands stimulation in human DCs. Thus, SOCS2 expression is specific and restricted to certain TLRs. Inhibition of NF-κB translocation eliminated SOCS2 protein expression after TLR4 signaling in DCs. Thus, we here identify the transcriptional factor NF-κB as the major SOCS2 inducer after TLR signaling.

Re-Programming of Tumor Derived T Regulatory Cells

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Sustained intratumoral IL-12 can restore cytotoxic function to tumor-associated CD8+ T effector/memory cells and induce the apoptotic death of Treg resulting in tumor regression. However, reversal of tumor immune suppression is transient and the tumors are rapidly re-infiltrated by Treg short-circuiting cytotoxic T cell activity. We predict that abrogation/delay of the Treg rebound will be critical to extending the antitumor effector window and achieving durable tumor regression. Based on recent reports demonstrating functional plasticity in CD4+ T cell subsets, we are testing strategies designed to re-program post-therapy Treg to T helper cells. One specific approach involves the local and

sustained delivery of siRNA and cytokines from controlled-release adjuvants. To determine whether Treg isolated from tumor draining lymph nodes (TDLN) could convert to Th17 cells, and establish the minimal cytokine requirement necessary for the phenotype switch, we performed in vitro culture assays. Foxp3 expression in tumor Treg cultured under Th17-polarizing conditions (TGF-B +IL-6) declined 1.5 fold. More importantly, the decrease of Foxp3 was paralleled with a similar fold increase in IL-17 expressing cells ($\sim$1.8 fold) indicating that both changes may be occurring within the same subset. These findings support the idea of plasticity of mature Treg isolated from TDLN and provide a basis for the in vivo application of polarizing conditioning as part of an immunotherapeutic approach. Separately, sustained high level Foxp3 expression is required for maintenance of Treg phenotype and function. Since the Foxp3 stability is implicated in CD4 T cell plasticity, the transient knock down of Foxp3 expression in tumor Treg by siRNA may play a pivotal role in driving the conversion of tumor associated Treg to Th17. Our preliminary results in splenocytes confirmed the successful use of the gold nanoparticles (GNP) for siRNA delivery with no apparent toxicity and efficient suppression of gene activity for up to 8 days. We are currently testing these formulations in combination to determine whether the phenotype of post-therapy Treg can be modulated in vivo.

A State of Dominant Tolerance is Rapidly Induced with Intravenous Dissemination but not with Subcutaneous Implantation in a Murine Leukemia Model

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While significant progress has been made in the identification of immune evasion mechanisms utilized by solid cancers, negative regulatory pathways employed by hematological malignancies have been under-explored. C1498 is an aggressive acute myelogenous leukemia (AML) cell line from C57BL/6 mice which we have transduced to express the model SIY peptide antigen. This model enables monitoring of endogenous $CD8^+$ T cell responses by specific tetramer and ELISPOT analyses, and also allows use of adoptively transferred 2C TCR Tg T cells to track specific responses. When implanted subcutaneously (SC) into syngeneic mice, C1498.SIY cells induced a robust functional $CD8^+$ T cell response as measured by SIY/K^b tetramer analysis and SIY-specific IFN-γ ELISPOT. In contrast, when injected intravenously (IV), a minimal SIY-specific immune response was detected. Interestingly, when C1498.SIY cells were injected IV 5 days prior to a subsequent SC C1498.SIY challenge, the SIY-specific T cell response was severely blunted, suggesting that IV tumor dissemination induces a state of dominant tolerance. This observation was unique to the IV setting, as a prior SC C1498.SIY challenge did not blunt but rather augmented the immune response to a subsequent SC C1498.SIY challenge. Tolerance induced by IV C1498.SIY appeared to be largely antigen-specific, as it did not significantly inhibit immune responses generated against OVA-expressing tumors. In order to dissect the mechanism(s) of tolerance induced by IV C1498.SIY, we employed adoptive transfer of CFSE-labeled 2C TCR Tg T cells. While similar numbers of 2C T cells had seen antigen as measured by expression of CD44, 2C T cells with IV tumor underwent significantly fewer cell divisions and were less functional upon restimulation compared with 2C T cells with SC tumor.

Interestingly, when C1498.SIY cells were administered IV first, prior to SC tumor implantation, CFSE dilution of transferred 2C T cells was severely blunted and associated with minimal accumulation of divided cells, consistent with cell death. Collectively, these results suggest that IV C1498.SIY may induce anergy and/or deletion of tumor-specific $CD8^+$ T cells, the final mechanism of which is currently being elucidated. Our results have implications for the future development of immunotherapy for patients with established disseminated leukemia in whom a dense state of tumor antigen-specific T cell tolerance may exist.

MHC Class II-Dependent Strain-Specific Differences in Phenotype and Function of Tumor-Infiltrating Myeloid Cells are Associated with Differential Outcome of TRAMP-PSA Tumor Growth in HLA-DR2b Transgenic Mouse Model

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We have recently demonstrated that MHC class II alleles can differentially affect anti-tumor immune responses and the outcome of tumor growth in HLA-DR2b transgenic mice (*J Immunol.* 2009;182:1242–46). The presence of a "permissive" HLA-DR2b allele in our model was associated with the development of strong antigen-specific antibody responses, suppression of CD8 T cell responses and enhanced growth of TRAMP tumor cells engineered to express prostate-specific antigen (PSA). In contrast, mice bearing a "non-permissive" I-A^b allele that did not support CD4 T cell responses to PSA, developed strong CD8 T cell responses in the absence of antibody responses, and rejected PSA-expressing tumors. We also analyzed a composition of the tumor-infiltrating leukocytes in DR2b+ and DR2b- mice bearing TRAMP-PSA tumors by flow cytometry. In DR2b+ mice, CD4 T cells, including CD25+foxp3+ regulatory T cells, were accumulated in larger number compared to DR2b-mice. The analysis of tumor-infiltrating myeloid cells also revealed significant stain-specific differences, particularly in the phenotype and activation status of CD11b+$Ly6C^{low}$ cells. $Ly6C^{low}$ cells accumulated in the tumors of DR2b+ mice had a different phenotype compared to DR2b-mice, and expressed higher levels of activation markers, most notably CD40. Although the function of CD11b+$Ly6C^{low}$ cells is poorly characterized, they are regarded as "stationary" monocytes with a potential to differentiate into CD8α-dendritic cells and cross-tolerize T cells through the programmed death ligand (PDL)-1. Due to their differential ability to process antigen, these cells were also reported to favor CD4 T cell activation. Up-regulation of CD40 on these cells observed in our experiments may serve as an indicator of their cross-talk with CD4 T cells at the tumor site, which is consistent with a "permissive" status of the HLA-DR2b allele. In contrast, tumor-infiltrating $Ly6C^{low}$ cells from DR2b-mice expressed low levels of CD40, which could indicate lack of a cross-talk between CD4 T cell and APC consistent with a "non-permissive" status of I-A^b allele. Based on our observations, we propose that a failure to reject TRAMP-PSA tumors in DR2b+ mice can be partially due to the significant presence of "stationary" tolerogenic $Ly6C^{low}$ myeloid cells that could preferentially interact with tumor-infiltrating CD4 T cells in an MHC class II-restricted Ag-specific manner, thus preventing somehow the effective terminal CTL differentiation.

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Human Myeloid Suppressor Cell Induction and Functional Analysis

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Tumor immune tolerance can derive from the recruitment of suppressor cell populations, including myeloid suppressor cells (MSC). MSC suppress anti-tumor T-effector responses through arginine depletion, reactive oxygen and nitrogen species production, and regulatory T cell expansion. In cancer patients and experimental tumor models, increased MSC correlates with more aggressive disease and poor prognosis. This study examined the ability of 100 human solid tumor cell lines to induce MSC using a novel in vitro tumor co-culture method. Newly induced MSC were characterized for suppression, morphology, phenotype, and gene expression. A group of 17 MSC-inducing cancer cell lines, as well as 6 non-MSC control cancer cell lines, was then evaluated for the production of putative MSC-promoting factors (TGFβ, IL-1β, IL-4, IL-6, IL-10, GM-CSF, M-CSF, IDO, FLT3L, c-kit L, TNFα, COX2, and VEGF) by ELISA and quantitative RT-PCR

techniques. To clarify the role of specific immune modulatory factors in the induction of suppressive function, 7 cytokines were tested for their ability to induce MSC from normal donor PBMC. Of 100 solid tumor cell lines examined (breast, cervical, ovarian, pancreatic, lung, head and neck, renal cell, liver, colorectal, prostate, thyroid, gastric, bladder, sarcomas, and glioblastomas), 46 generated $CD33^+$ cells with suppressive capacity sufficient to inhibit autologous $CD8^+$ T cell proliferation by at least 20% at a 1:4 ratio. Tumor cell line-induced MSC were $CD33^+CD11b^+CD66b^+HLA\text{-}DR^{var}IL\text{-}13R\alpha2^{int}$ large mononuclear cells with abundant basophilic cytoplasm and had strongly upregulated expression of iNOS, TGFβ, NOX2, VEGF, and ARG-1, consistent with human MSC derived from cancer patients. MSC were generated with high frequency by cervical (4/5) and ovarian (8/9) cancer cell lines, but 0/9 breast cancer cell lines examined. Comparison of tumor derived factors expressed by these cell lines supported multiple pathways for MSC generation, including IL-1β, IL-6, and COX2. Subsequent in vitro cytokine induction studies demonstrated that IL-1β, IL-6, (COX2-derived) PGE2, TNFα, and VEGF with GM-CSF, but not TGFβ, are sufficient for the induction of suppressive $CD33^+$ MSC from normal donor PBMC. Human MSC had increased expression of iNOS, TGFβ, NOX2, VEGF, and ARG-1 relative to non-suppressive myeloid cells, though gene expression varied with the inducing cytokine(s). Expression of inhibitory ligands PDL1, PDL2, or B7H4 by human myeloid cells did not correlate with suppressive function, nor did expression of transcription factor CCAAT/enhancer binding protein β, which has been suggested as a murine MSC marker. These data have implications for the development of MSC-targeted therapies for the immunotherapy of cancer and provide a novel method of human MSC generation by tumor co-culture.

IL-6 and IL-10 are Correlated with Distinct MDSC Profiles in Patients with Gastrointestinal Malignancy

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IL-6, IL-10 and IL-1b are mediators of immunosuppression in a tumor-bearing host. These cytokines can regulate the survival and function of myeloid-derived suppressor cells (MDSC). We studied the relationship between circulating cytokines and MDSC in a cohort of n = 40 patients with gastrointestinal (GI) malignancy. Most patients (77.5%) had stage III or IV disease, Eleven (27.5%) had prior therapy , and 13 (32.5%) had prior surgery. Plasma IL-6 and IL-10 were elevated in patients versus normal donors. CD33+HLADR-CD11b+CD15+ and CD33+HLADR-/lowCD14+ MDSC were elevated in patients versus normal donors ($P < 0.0001$). Prior studies have suggested that the CD15 marker divides human MDSC into subsets with different suppressive functions. Thus we evaluated the relationship between CD15 expression on MDSC and cytokines elevated in GI patients. A correlation was evident between IL 6 and CD15+ MDSC ($P = 0.0107$), but not $CD15^-$ MDSC ($P = 0.163$) or CD14+ MDSC ($P = 0.218$). In contrast, IL-10 correlated with the $CD15^-$ MDSC ($P = 0.003$) but not the CD15+ MDSC ($P = 0.320$) or CD14+ MDSC ($P = 0.587$). Plasma IL-1b did not correlate with any MDSC subset. These data suggest a unique relationship between circulating MDSC and cytokines in patients with GI malignancy. The relationship between overall survival (OS) and MDSC subsets was next analyzed. The hazard of death was unrelated to MDSC subset in a univariable Cox proportional hazards model (CD14+ $P = 0.179$; CD15+ $P = 0.347$; $CD15^-$ $P = 0.318$). However, in a multivariable model including $CD15^-$ and $CD15^+$ MDSC, the hazard ratio (HR) increased with percentage of CD15- MDSC but decreased with increasing percentage of CD15+ MDSC (HR for 2-fold increase in CD15− = 1.42, $P = 0.038$; HR for 2-fold increase in CD15+ = 0.71, $p = 0.038$). Because prior studies have highlighted the importance of the CD15 marker in defining human MDSC subsets, we considered if the proportion of CD15− to CD15+ MDSC might reflect the clinical profile of patients. The relationship between OS and the log of the ratio of CD15− and CD15+ MDSC was evaluated. The HR significantly increased with an increased ratio of CD15− and CD15+ MDSC (HR = 1.42 for a 2-fold increase in CD15− relative to CD15+; $P = 0.027$). Interestingly, adding stage of disease or number of systemic therapies into this model did not alter the HR of the CD15−/CD15+ ratio, indicating these factors did not confound the relationship between the ratio and survival. Finally, greater CD15− as compared to CD15+ MDSC was more likely in patients with stage III or IV cancer. These data reveal the presence of a unique relationship between systemic pro-inflammatory cytokines, MDSC phenotype and potentially disease stage or OS in patients with GI malignancy.

Evaluation of Myeloid and Lymphoid Regulatory Cell Populations in the Peripheral Blood of Patients Receiving GM-CSF in a Vaccine-Based Immunotherapy Clinical Trial

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In a recent multipeptide vaccine trial for melanoma, patients receiving GM-CSF had a diminished circulating CD8 T cell response to the vaccine compared to those without GM-CSF. Other studies have implicated a mature myeloid population in a decreased immune response in patients receiving a vaccine with GM-CSF. We investigated whether changes in this population or other selected regulatory cell populations in the peripheral blood may explain the decreased CD4 and CD8 T cell responses in patients receiving GM-CSF compared to those who did not. All patients had received the MELITAC 12.1 vaccine (12 melanoma peptides plus tetanus helper peptide, in incomplete Freund's adjuvant with or without GM-CSF). After completion of the vaccines, patients receiving GM-CSF had lower immune responses to

TABLE 1. Myeloid Cell Populations Do Not Increase Proportonately in the Peripheral Blood of Patients Receiving GM-CSF as Adjuvant Therapy in Melanoma Patients

		Percent of Viable $CD45^+$ Nonlymphoid Cells (Lineage Negative for CD3, CD19, CD56)					
		No GM-CSF			GM-CSF		
Cell Phenotype	Timing re:vax	Mean	Median	*P*	Mean	Median	*P*
$CD14^+$ DR^-/lo	Pre	4.0	1.2	0.94	2.9	2.9	0.81
	Post	3.8	1.7		2.7	1.7	
$CD11b^+$ $CD33^+$ HLA^- DRneg	Pre	2.8	0.8	0.77	2.0	1.5	0.70
	Post	2.3	0.6		2.2	2.0	
$CD33^+$ $CD15^+$	Pre	5.1	1.1	0.92	3.3	1.6	0.90
	Post	4.8	1.4		3.5	2.1	

Statistical significance as determined by a 2-tailed Student's *t* test assuming equal variance between 2 samples.

the peptides incorporated in the vaccine as measured by ELISpot, and by MHC/multimer reagents. Myeloid populations were identified by flow cytometry, as a proportion of CD45+, lineage (CD3, CD19, CD56)-negative cells. Mature myeloid suppressor cells (MSC) were further defined as $CD14^{+}$HLA-DR < lo/neg >, and myeloid-derived suppressor cells (MDSC) of two phenotypes were defined as $CD11b^{+}CD33^{+}$DRneg or $CD33^{+}CD15^{+}$. Regulatory T cell populations also were defined as CD25bright FoxP3+ CD4+ by multi-parameter flow cytometry. Twenty patients were evaluated, half in each group, pre and after 4 vaccines. After four weeks of weekly immunizations, the level of MSC did not change significantly (median 1.7% after vaccine in both groups, Table 1 previous page). In addition, other myeloid populations did not change significantly after immunization + GMCSF ($P = 0.7$, 0.9, Table 1 previous page) and were not different between groups (Table 1 previous page). Also, there was no overall change in regulatory T cells in patients receiving GM-CSF ($P = 0.49$, not shown). The suppressed immune responses associated with GM-CSF in this trial do not appear to be attributable to any of these defined regulatory cell population in lymphoid or myeloid compartments of peripheral blood, but may operate by other mechanisms that impact the immune response to tumor-derived peptides.

Blockade of TGF-β Receptor Type I Signaling Reduces the Ability Of FoxP3+ Cells to Inhibit Proliferation and IFN-Gamma Production in Effectors Cells

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Transforming growth factor-beta (TGF-β) plays a crucial role in tumor growth, metastasis and invasiveness. We already demonstrated that the beneficial effect of systemic blockade of TGF-β receptor type 1 by a small molecule inhibitor (SM16) in a murine breast cancer model is T cell dependent. As TGF-β is capable of redirecting peripheral CD4+ T cells toward a FoxP3+ Treg phenotype, which contribute to immune suppression and tumor evasion, the current studies were designed to evaluate how the disruption of TGF-β signaling affects Treg function and contributes to the antitumor effects of SM16. We demonstrate that systemic blockade of TGF-β receptor type I by SM16 is insufficient to prevent expansion of the Treg compartment. Instead, SM16 induced expansion of both Treg (CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+) and effector cell compartments in naïve and tumor bearing mice (TBM). Strikingly, the SM16-induced expansion of FoxP3+ cells was associated with reduced ability to inhibit proliferation of effectors in in vitro suppression assays. Finally, SM16 impaired the ability of FoxP3+ cells to suppress IFN-γ production by CD4+CD25-FoxP3- effector T cells. In summary, our data suggest that SM16 may promote tumor immunity by dampening the ability of FoxP3+ cells to inhibit proliferation and IFN-γ production in effectors cells.

Chronic Chemoimmunotherapy Maintains Long-Term Anti-Tumor CD8+ T Effector Cell Activity and Results in the Cure of Advanced Spontaneous Tumors

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Like other forms of cancer immunotherapy, intra-tumoral delivery of microsphere encapsulated IL-12 and GM-CSF leads to only short-lived tumor regression in the Her-2/neu spontaneous mammary tumor model. IL-12/GM-CSF treatment promotes an initial loss of T regulatory cells from tumors which corresponds with tumor regression. However, T regulatory cells inevitably re-infiltrate the tumor and tumor growth resumes as CD8+ T effector cells are rendered nonfunctional. With multiple intratumoral IL-12/GM-CSF treatments, the T-regulatory cell rebound is progressively exacerbated and tumor specific CD8+ T cells are lost. To circumvent this counter-regulatory response we have specifically targeted the T-regulatory cell re-infiltration of the tumor and draining lymph nodes. Low-dose chemotherapy has been associated with depletion of T-regulatory cells and has been shown to enhance tumor immunotherapy. In our studies repeated administration of cyclophosphamide and IL-12/GM-CSF microspheres over a period of nine weeks led to complete and durable regression of spontaneous tumors, while either treatment alone was ineffective in achieving long-term cure. Importantly, whereas contribution of CD8+ T-cells to tumor regression in the immunotherapy alone group was minimal, tumor kill was strictly dependent on CD8+ T-cells in the chemoimmunotherapy group. Further analysis revealed that chemoimmunotherapy enhanced both the proliferation (2-fold) and the cytotoxic activity of CD8+ T cells (up to 4.7 fold) over that of immune therapy alone as assessed by BrdU uptake and granzyme B, perforin, and IFN-γ expression. Ultimately, persistent suppression of the post-therapy T regulatory cell rebound led to an elevated activity index (Proliferating CD8+ T cells: T regulatory cells) in the tumor, which correlated with tumor regression. These studies demonstrate that homeostatic counter-regulation, a major challenge of immune-based cancer therapies, can be overcome by chronic chemoimmunotherapy.

CD4+Foxp3+ Regulatory T Cells are Dependent on PI3K Pathway Allowing for Their Selective Inhibition

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Naturally-occurring Regulatory T cells (Tregs) mediate tumor immune-evasion. Their depletion or inactivation increases anti-tumor responses and decreases tumor burden. In this study, we identified the differences in the role of PI3K/Akt pathway in T cell receptor signal transduction in Tregs and conventional T cells (Tconv), and exploited such differences to selectively deplete Tregs in vivo, while keeping other T cell subsets unaffected. We found that unlike Tconv, Tregs are mainly dependent on the PI3K-Akt pathway for polyclonal signaling downstream of the T cells receptor (TCR) in vitro. Inhibiting the PI3K-Akt pathway by small molecule inhibitors in Tregs decreased S6 phosphorylation, and completely abrogated their proliferation in response to TCR/CD28/IL-2 stimulation. In contrast, these inhibitors had minimal effects on S6 phosphorylation and proliferation in Tconv cells. In vivo, administering PI3K or Akt inhibitors preferentially reduced the number of Tregs and Foxp3 mRNA, increased specific CD8 T cell immune responses to vaccination, and resulted in decreased Treg-dependant tumor growth, which was reversed after re-infusing Tregs, indicating that inhibitors of PI3K and Akt directly target Tregs in vivo. Accordingly, we conclude that TCR/CD28/IL-2 signaling is mainly dependent on PI3K pathway in Tregs, but not in Tconv cells, and inhibiting PI3K and Akt directly target Tregs both in vitro and in vivo. This forms the basis for a clinically-relevant selective Treg cell inhibition strategy in vivo using small molecule inhibitors.

Assessing and Countering Negative Immune Regulation in Renal Cell Cancer Patients-Results of a Randomized Phase II Trial with IMA901

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Background: IMA901 is a therapeutic cancer vaccine for the treatment of renal cell cancer patients based on the selection of naturally presented tumor-associated peptides (9 HLA-class I- and 1 HLA class II-binding peptides). A previous phase I study

(N = 28 patients) showed significant correlations of clinical benefit with T-cell responses to multiple IMA901 peptides and between pre-vaccine levels of Foxp3+ regulatory T cells (Tregs) and immune responses. Based on this experience, a randomized phase II study was designed to explore the biological and clinical efficacy of IMA901 with or without CY as an immunomodulator. Different regulatory cell populations in patients prior to vaccination were also assessed.

Phase II study IMA901-202: A total of 68 HLA-A*02-positive RCC patients with documented progression during or after first-line therapy were randomized to receive or not a single infusion of low-dose cyclophosphamide (CY; 300 mg/m^2) three days prior to the start of repeated i.d. vaccinations with IMA901 with 75 μg GM-CSF i.d. 64 patients were eligible according to the pre-specified per-protocol analysis. Patients receiving a single infusion of CY showed a strong trend for improved overall survival (OS) as compared to the non-CY group (OS not reached after 25 mo of follow-up vs. 16 mo, respectively; $P = 0.086$). CY-pretreatment resulted in a significant decrease of Tregs after 3 days, most prominently of proliferating Ki67+ Tregs ($P = 0.006$). Marginally significantly better OS rates were seen in immune responders compared to non-responders ($P = 0.048$), but highly significantly so in patients randomized to CY ($P = 0.006$).

Several cellular biomarkers associated with immune regulation were assessed prior to the start of vaccination: four populations of myeloid-derived suppressor cells (MDSC) described in the literature, IL-10- and IL-17-secreting CD4 T cells and dysfunctional T cells (measured by decreased TCR zeta expression) were all highly significantly ($P < 0.0001$) elevated in RCC patients compared to matched healthy donors. Two MDSC populations were significantly (negatively) associated with survival ($P = 0.033$ and $P = 0.005$).

Conclusions: To our knowledge, this is the first randomized study assessing the impact of low-dose cyclophosphamide on FoxP3-positive regulatory T cells in patients demonstrating that CY predominantly acts on proliferating Ki67+ Tregs. Furthermore, this study demonstrates highly significantly elevated levels of MDSC, IL-17-/IL-10-secreting T cells and dysfunctional T cells and a significant negative correlation of MDSC with overall survival in RCC patients.

A Single Dose of Ontak Administration is Not Sufficient to Deplete Tregs in Stage IV Melanoma Patients

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CD4+CD25+FoxP3+ Tregs have been shown to be expanded in a subset of melanoma patients. We recently have observed that Tregs are particularly enriched in melanoma metastases that are inflamed and contain CD8+ T cells. These data suggest that Tregs might suppress the activation and function of tumor-reactive CD8+ effector T cells in the melanoma tumor microenvironment, arguing that Treg depletion might have therapeutic benefit. Ontak is a recombinant fusion protein between IL-2 and diphtheria toxin, which can bind to CD25-expressing cells, leading to internalization and subsequent cell death. In a previous clinical study in renal cell carcinoma patients, a single intravenous dose of Ontak administration resulted in significant reduction of CD4+CD25high Treg numbers in the peripheral blood and enhanced stimulation of cytotoxic T cell responses. However, in another clinical trial in stage IV melanoma patients, a multiple dose regimen of Ontak failed to deplete Tregs in the peripheral blood. Based on these conflicting results, we investigated whether a single dose of Ontak treatment could effectively deplete Tregs in stage IV melanoma patients, prior to a multipeptide vaccine. In a randomized phase II design, 21 patients were randomized to receive a 4-peptide vaccine in Montanide and GM-CSF, with or without administration of a single dose of Ontak (18 mcg/kg) on Day -4. Six patients were randomized to the Ontak arm. PBMC were analyzed before and 3 days after Ontak administration. Tregs were stained with CD4, CD25, and intracellular FoxP3 antibodies, acquired on an LSR II instrument, and analyzed using FlowJo Software. Contrary to expectations, there was no significant decrease in circulating Treg numbers between pre- and post-treatment samples (pre: 24.4 ± 11.06/μL; post: 23.7 ± 14.62/μL; $P = 0.9021$). In one patient, tumor biopsy material was available pre-treatment and at week 5 following treatment, which showed that FoxP3 transcripts were increased in the post-treatment sample. We conclude that a single dose Ontak is not sufficient to meaningfully deplete Tregs in melanoma patients. Whether vaccine-induced CD8+ T cell responses might have been augmented is currently being evaluated. Alternative approaches to deplete Tregs should be pursued.

DENDRITIC CELLS AND CANCER

DCs Matured With LPS/PGE_2 Induce the Direct Differentiation of Naïve $CD8^+$ T Cells into Central-Memory Cells

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Trials of cancer vaccines have shown that the induction of high numbers of circulating tumor-specific $CD8^+$ T cells is not necessarily accompanied by acquisition of an effector function. On the other hand, it was shown that in the absence of memory cells, adoptively transferred effector T cells mediate only transient therapeutic effect and rapidly disappear, being unable to prevent disease recurrence. While the success of vaccination and other forms of immunotherapy of cancer and chronic infections is believed to depend on successful induction of both memory and effector $CD8^+$ T cells, the signals that drive the differentiation of $CD8^+$ T cells into each of these subsets, and the exact mechanism of their development (sequential development of effector and memory cells versus independent parallel development of effector and memory cells) remain unclear.

We show that dendritic cells (DCs) matured in the conditions of acute/early inflammation (LPS/IFNγ) induce the expansion of $CD8^+$ T cells and their acquisition of CTL phenotype ($GrB^{high}CCR7^-$) and functions, followed by development of effector-memory ($GrB^{low}IL\text{-}7R^+IL\text{-}15R^+CD62L^-CCR7^-$) and central-memory ($GrB^{low}IL\text{-}7R^+IL\text{-}15R^+CD62L^+CCR7^+$) cells. In contrast, DCs matured in the presence of LPS and PGE_2 (a mediator of late/chronic inflammation) instruct $CD8^+$ T cells to directly develop into central-memory cells without passing through an effector stage with cytolytic function. Analysis of the RNA expression of selected genes showed that the LPS/PGE_2-DC-primed $CD8^+$ T cells have the same expression pattern as blood-isolated central-memory cells. Similar to blood-isolated central-memory $CD8^+$ T cells, the non-cytolytic $CD8^+$ T cells induced by LPS/PGE_2 matured DCs rapidly acquire CTL function upon secondary interaction with type-1 polarized DCs, indicating that they are fully functional.

The specialized functions of DCs matured in the conditions of early versus late/chronic inflammation as selective inducers of the effector/effector-memory versus central-memory pathways of $CD8^+$ T cell differentiation help to explain the preferential induction of memory $CD8^+$ T cells at late stages of immune responses, and provide for new strategies of enhancing the effectiveness of immunotherapies of cancer and chronic infections.

Dentritic Cell-Based Cancer Therapeutic Vaccine Targeting HAAH Using Nano-Particles to Overcome Tolerance and Avoid Auto-Immune Response

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Utility of autologous dendritic cells (DC), maturated ex-vivo and loaded with cancer associated antigens (CAA) is well established as therapeutic vaccine. Other than choice of stimulants the CAA and

its presentation to DC is critical in overcoming self antigen tolerance and avoiding autoimmune response. We are developing a DC based vaccine therapy protocol targeting HAAH with a novel approach of using nano-particles to introduce HAAH to the professional antigen presenting cell. As the target, HAAH is a tumor specific antigen which is specifically expressed on the surface of malignant cells. It is responsible for proliferation, motility and invasive phenotype of cancer cells. HAAH internalizes when bound to the antibodies. Neutralizing or inhibiting expression of the cell-surface HAAH, reverses cancer cell to normal phenotype. Anti-HAAH antibodies have proven efficiency in inhibiting tumor growth in animal studies (passive immunotherapy). Aside from the target, the loading of DC is accomplished by utilizing nano-particles with HAAH fragments on their surface. The technique is novel and consists of engineering lambda-bacteriophage to express HAAH-constructs on their surfaces. We have been successful in purifying this baceriophage and neutralizing them to become non-replicating particles. These particles have 200 to 300 copies of HAAH fragments on their surface and their CpG motifs in DNA sequence act as an excellent adjuvant and stimulant. Delivering HAAH antigens on lambda head will help to induce co-stimulatory activity in antigen presenting cell such as dendritic cells, during processing of foreign antigens on those cells. Thus in this case such added lambda protein and its CpG moiety act as an adjuvant. Also due to particulate nature, phage-displayed HAAH peptides can access maturation through both the major histocompatibility complex (MHC) I and MHC II pathway, and activates both T cell and B cell mediated immunity against HAAH specific cancer antigens. The added cellular response mediated by CD8+ T cells helps to eliminate cancer cells by active lysis process. The non-typical presentation of the antigen and its specificity to cancer cells results in overcoming the self antigen tolerance an un-wanted auto-immune response. The first indication is lung cancer for which we have an HAAH-based companion diagnostic test for enrolment and monitoring.

Global Gene Expression Analysis of 15 kD Granulysin Stimulated Monocytes Compared to GM-CSF

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Granulysin is an immunoregulatory protein existing in several isoforms. The 9 kD isoform is proinflammatory, chemotactic and cytotoxic against microbes and tumors. The 15 kD isoform is not well characterized functionally although its expression plays a key role in some excessive immune reactions. Unpublished data from our laboratory indicates that the 15 kD form induces the differentiation of monocytes into immature dendritic cells (DCs). The aim of this study was to characterize at the transcriptome level the effects of 15 kD granulysin on monocytes. RNA of elutriated monocytes from three donors cultured for 4, 12, 24 and 48 hours in presence of GM-CSF or 15 kD granulysin was amplified, labeled and hybridized on Agilent Oligo Microarrays. Gene expression analysis revealed a common cytokine-induced signature at each time point, with a more specific signature for each cytokine at later time point. GM-CSF specifically downregulated genes related to cell cycle arrest and the immune response. In particular, cytokine production, lymphocyte mediated immunity and humoral immune responses were the Gene Ontology families overrepresented among the downregulated genes at late time points. On the other hand, 15 kD granulysin induced immune response, chemotaxis and cell adhesion genes. Moreover, only 15 kD granulsyin induced the activation of pathways related to fundamental DC functions, such as co-stimulation of T cell activation and Th1 development. This study provides important insights on the effects of a novel agent, 15 kD granulysin, that will likely be useful for future immuno-therapies aimed at activation of the immune response (Fig. 1).

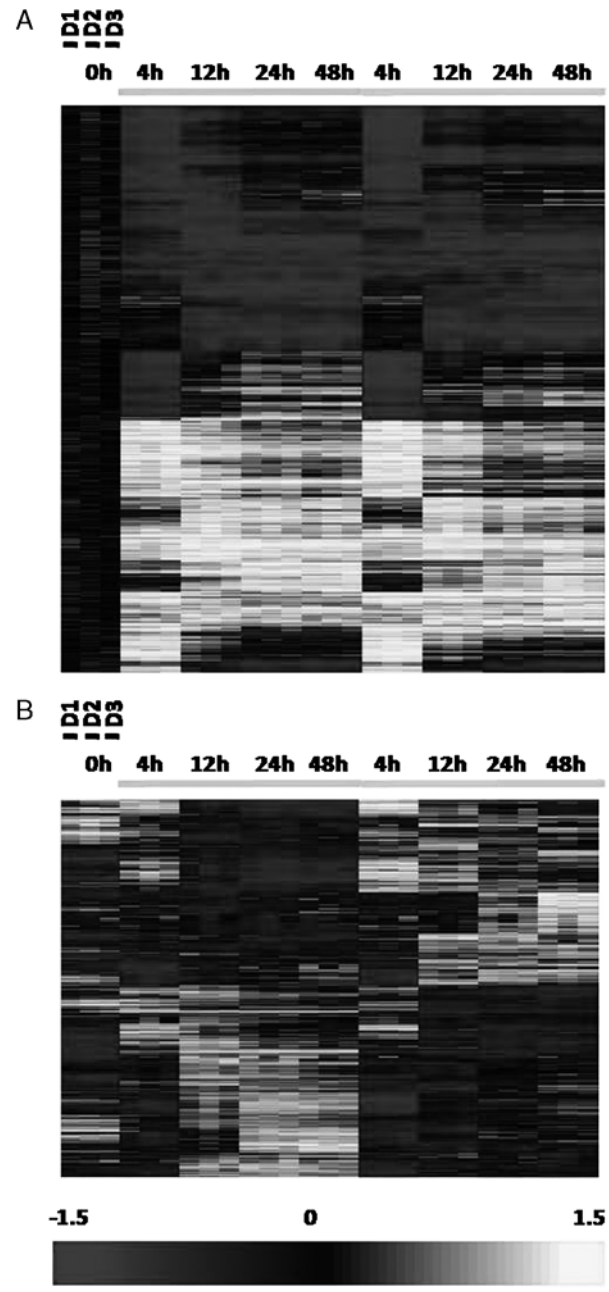

FIGURE 1. A, Heat map of 6103 genes statistically differentially expressed ($P<0.001$) by GM-CSF and 15 kD granulysin compared with untreated monocytes. B, Heat map of 3690 genes statistically differentially expressed ($P<0.001$) between GM-CSF and 15 kD granulysin. Orange bar identifies GM-CSF-activated monocytes, whereas green bar indicates 15 kD granulysin-activated monocytes.

Resistance to the Proapoptotic Effects of IFN-γ on Melanoma Cells Used in Patient-Specific Dendritic Cell Immunotherapy is Associated with Improved Overall Survival

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The use of whole cell tumor vaccines and various means of loading antigen onto dendritic cells have been under investigation for over

a decade. Induction of apoptosis and the exposure of immune stimulating proteins are thought to be beneficial for use in immunotherapy protocols but conclusive evidence in the clinical setting has been lacking. Incubation of commercially available melanoma cell lines (A375, SK-MEL-5, SK-MEL-28) with interferon-gamma (IFN-γ) increased phosphatidylserine (a measure of early apoptosis) and calreticulin exposure but not in the interferon-gamma resistant cell line (Lu-1205). Short term autologous melanoma cell lines used for loading dendritic cells for immunotherapy showed differential response to the pro-apoptotic effects of IFN-γ.

These IFN-γ treated tumor cells were irradiated and used for loading antigen for dendritic cell therapy. A log-rank comparison of survival for patients whose tumor cells were found to be either sensitive (up-regulated phosphatidylserine and calreticulin) or insensitive to IFN-γ, revealed a strongly significant correlation to progression-free ($P = 0.003$) and overall survival ($P = 0.002$) favorably in those patients whose cell lines were resistant to the proapoptotic effect of IFN-γ. A remarkable 10/23 patients in the insensitive cohort were still live after 60 months of follow up with 6 of those still remaining disease free. These results suggest that the use of IFN-γ in anti-melanoma dendritic cell-based immunotherapy may only be beneficial if the cells do not undergo apoptosis in response to IFN-γ and support the contention that the use of some apoptotic cells in vaccines may be detrimental.

Features Associated with Survival in Metastatic Melanoma Patients Treated with Patient-Specific Vaccines Consisting of Proliferating Autologous Tumor Cells and Autologous Dendritic Cells

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Background: We previously reported a 54% survival rate at a median follow up of 4.5 years for 54 metastatic melanoma patients enrolled in an open-label phase II trial in which they were treated with patient-specific vaccines consisting of autologous dendritic cells loaded with antigens from short-term autologous tumor cell lines (Cancer Biother Radiopharm 24:311 to 319, 2009).

Methods: The dependent variable of interest was survival after initiating vaccine therapy. Multiple univariate analyses identified factors associated with significant differences in log rank tests of survival curves ($P^2 < 0.05$). Multiple linear regressions were developed using a process of stepwise regression for the dependent variable of survival at 3.5 years.

Results: Minimal follow up for surviving patients is now 3.7 years, median follow up 5.5 years, and 5-year survival rate is 50%. Of 26 features documented for all 54 patients, 6 were associated with improved survival by univariate analysis: ECOG performance status (PS) of 0, not having measurable disease at the time of vaccine therapy, receiving the planned 8 vaccinations, age < 50 years, having a baseline serum lactate dehydrogenase (LDH) in the normal range, and having had M1a or M1b metastases as opposed to M1c disease. Additional features that were not available for all patients, but associated with improved survival, were failure of gamma interferon (g-IFN) to induce apopotosis in the autologous tumor cells, and anergy to baseline standard skin tests for Candida or Trichophyton. Using variables for which information was complete for all 54 patients, the best multivariate regression for classification of survival at 3.5 years (32 alive, 24 dead) utilized 6 independent factors: prior radiation therapy, age, gender, ECOG PS, and numbers of all cells and viable cells administered in the first 3 injections. This model correctly identified death by 3.5 years for 20/22 patients (91%), and correctly identified survival for 28/32 (87%). When using the incomplete data set (n = 49 patients) the best multivariate regression included these same 6 features plus g-IFN-induced apoptosis. This 7-feature model correctly identified death by 3.5 years for 19/20 (95%) while being alive at 3.5 years was correctly indentified for 27/29 (93%).

Conclusions: Most of the variables associated with longer continuous survival per univariate analysis are not independent of one another. We identified a multivariate linear model that combines 7 independent clinical and tumor cell-related features with higher classification accuracy for survival at 3.5 years than any single feature or other combination of features.

Dendritic Cells Transfected with mRNA for p53, Survivin and hTERT as Treatment for Patients With Malignant Melanoma or Breast Cancer-A Phase I Study

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Cancer vaccines based on peptide pulsed dendritic cells (DC) have been tested in several clinical trials. One of the shortcomings of peptide pulsed DC vaccines is the HLA -restriction of the tumor associated antigens (TAA). One way to overcome this restriction is by transfection with mRNA into the DC by electrophoresis. Thereby, the protein is synthesised, degraded and presented by the HLA molecules in a more natural way and can potentially lead to a broader and more potent immune-response (polyclonal response) independently of a HLA-restriction. Based on our preclinical studies in breast cancer patients which showed both CD4+ and CD8+ TAA specific T cells after in vitro stimulation with p53-, surivivin-, and hTERT transfected autologous DCs we have initiated this clinical phase I study with transfected DCs.

Methods and materials: Patients with progressive advanced malignant melanoma or breast cancer receive intradermal injections with autologous DCs transfected with mRNA for survivin, hTERT and p53. DC injections are given every second week hereafter every four weeks until progression. Concomitant with DC therapy patients are treated with metronomic cyclophosphamide (Cy) 50 mg × 2/d for a week, every second week. Immunological response is measured in peripheral blood-samples at baseline, at the 4th and 6th vaccines and, if the patient achieve SD, every 3rd month. Clinical evaluation according to RECIST criteria is performed every 3rd month.

Results: The planned 14 patients are included and treatment is ongoing. Data regarding toxicity and clinical response will be available. Furthermore, results from ongoing analyses of immune parameters including evaluation of induced vaccine specific immunity and regulatory T cells will be presented.

Conclusion: Safety and tolerance are primary aims in this study. Secondary, objective responses, specific T cell responses and changes in Tregs will be measured.

Combined Treatment with Dendritic Cell Vaccine and Low-Dose Cyclophosphamide in Patients with Malignant Melanoma

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Background: Cancer vaccines based on dendritic cells (DC) have been tested in several clinical trials but in general, efficacy has been low. The inhibitory effect of regulatory T cells (Tregs) on the immune system and the effect of chemotherapy on the number and function of Tregs are intensively investigated. Here we describe the clinical outcome and changes in Tregs in patients treated with DC and cyclophosphamide (Cy).

Methods and materials: Patients with progressive advanced melanoma received treatment with autologous DCs pulsed with p53, survivin and telomerase derived peptides (HLA-A2+) or tumor lysate (HLA-A2−). Metronomic Cy 50 mg × 2 daily for a week, every second week, celecoxib (Ce) 200 mg daily continuously, and IL-2 2 MIU day 1 to 5 following each vaccine, were given concomitantly. Vaccine no. 1 to 4 was given weekly and vaccine 5 to 10 biweekly. If the patient achieved stable disease (SD) vaccines were continued monthly, until progression.

Results: 17 of 28 patients (60%) achieved SD after 6 vaccinations and 8 of these (29%) maintained SD after 10 vaccines (4 mo). One patient has ongoing SD for 20 vaccines (13 mo). No objective responses were seen. Two patients were excluded prior to the first evaluation due to progression and 1 was excluded after 4 vaccines due to anemia and refusal of blood transfusion. One patient did not receive any vaccines due to rapid progression and death and is not included in the results. The treatment was associated with a marked increase in the proportion of Tregs from baseline to the 4th vaccine followed by a decrease from the 4th to the 6th vaccine although not to the baseline values.
Conclusion: The combination of DC vaccination and Cy was well tolerated. The induction of Tregs was not decreased by adding Cy, on the contrary a higher Treg level was maintained. However, the fraction of patients who achieved SD was more than doubled as compared to a comparable cohort of patients treated with DC vaccination without Cy. The fact that there is no concordance between the increase in Tregs and the beneficial outcome, outline the complexity of the in vivo immunology. Evaluations of vaccine specific immune responses are ongoing.

Dendritic Cell Vaccination in Cancer: Achievements, Obstacles and Future Perspectives

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We exploit dendritic cells (DCs) to vaccinate melanoma patients. We recently demonstrated a statistical significant correlation between favorable clinical outcome and the presence of vaccine-related tumor antigen specific T cells in delayed type hypersensitivity (DTH) skin biopsies. While we find immunological responses in 30% to 50% of the patients, favorable clinical outcome is only observed in a minority of the treated patients. Therefore, it is obvious that current DC-based protocols need to be improved to increase clinical efficacy. For this reason, we study in small proof of principle trials the fate, interactions and effectiveness of the injected DCs.
We compared DC loaded with tumor antigen specific MHC class I binding peptides alone, in combination with MHC class II binding peptides or with defined tumor antigen mRNA (gp100 and tyrosinase). The results show that the presence of supplementary tumor antigen-specific MHC class II epitopes result in an T helper response that might be beneficial for the clinical outcome in these patients. Furthermore, comparing different routes of administration we observed that intranodal injection is not always successful (MRI) and that only a small proportion of the intradermally administered DCs reach the lymph nodes (scintigraphy). Our data clearly indicate that the cells that reach the lymph nodes are fully mature DCs that are able to induce an immune response in vivo.
We have begun to explore the potency of DC subsets in the peripheral blood. Recently we have completed a phase I trial with plasmacytoid DCs. Results will be discussed as well as the potency of other DC subsets.
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Combined Intraprostatic Autologous Dendritic Cell Injection with Radiation Therapy in Localized, High Risk Prostate Cancer: Serial Assessment of Apoptosis and Lymphocyte Infiltrates

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Previous work in murine models suggested combining local radiotherapy (XRT) with intratumoral syngeneic dendritic cell (DC) injection could result in apoptosis/cell death mediated effective induction of cytotoxic T lymphocytes (CTL) based anti-tumor immunity. However, the presence, timing, and effectiveness of effector cell infiltrates prior, during, and following combined XRT/intratumoral DC injection in humans is unknown. Herein, we report the feasibility and tolerability of intraprostatic autologous dendritic cell injection; five HLA-A2+, high risk localized prostate cancer patients were treated on an investigational protocol using androgen suppression therapy, external beam radiation therapy (EBRT, 45 Gy) followed by brachytherapy permanent interstitial implant with addition of experimental autologous intraprostatic DC injections during EBRT, after fractions 5, 15, and 25 of 25. Multiple serial prostate biopsies were collected before initiation of treatment, during EBRT and at 3, 12, 24 & 36 months after completion of treatment. Biopsies were stained for hematoxylin and eosin, CD4, CD8, and cleaved caspase 3, and evaluated in a blinded manner. The specific anti-tumor immunity was assessed via ELISpot interferon gamma production by lymphocytes stimulated by HLA-A2 peptides derived from sequences of proteins associated with prostate cancer. Apheresis, intraprostatic DC injections, and biopsies were well tolerated. The pattern of distribution of CD8+ cells was consistent with prostate cancer antigen targeting, rather than non-specific organ infiltration. There was no immediate obvious intraprostratic infiltrate by effector cells after DC injection in vivo, in humans. Measurable, induced increases in ELISpot titers in peripheral blood CTL were observed for some subjects, for some antigens, but non-specific immunity also fluctuated. This initial translational experience demonstrates safety of intraprostatic injection of autologous DC injection coordinated combined modality hormone suppression and radiation. Design of future trials employing combination XRT and intratumoral DC injection should consider appropriate timing concerns to match therapy-induced apoptosis with the timing of dendritic cell injections.

Dendritic Cell and Myeloid Derived Suppressor Changes with Interleukin-2 Therapeutic Administration

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High-dose intravenous interleukin-2 (IL-2) therapy (14 doses/course, 2 courses/cycle) for metastatic melanoma or kidney cancer (RCC) can induce major responses; subcutaneous IL-2 has been also used in RCC therapy. In three concurrent clinical trials, we evaluated (N = 45) the effect of IL-2 on dendritic cell (DC) and myeloid derived suppressor cells (MDSC). The first intervention employed a new schedule (dose of 600,000 IU/kg, 8 h between doses, 5 doses/course, 4 courses at weekly intervals per cycle) of high-dose IL-2; the second used all trans retinoic acid (ATRA) followed by subcutaneous IL-2 in RCC; and the third used intravenous bevacizumab followed by subcutaneous IL-2 in RCC. Herein, we report a hypothesis-generating observation that the patients treated with high dose IL-2, who had most favorable outcomes had high (88.8, median) pre-treatment DC-to-MDSC ratios, similar to the pattern in healthy individuals. However, the DC-to-MDSC ratio, even in those for whom it was favorable at the outset declined (20.1, median). A general decline of DC-to-MDSC ratio also was observed on low dose IL2 combination regimens (N = 29). Thus, changes induced by IL-2 in the MDSC number and the DC-to-MDSC ratio merit further detailed clinical and laboratory evaluation; pretreatment assessment of DC phenotypic status may be a starting point for patient selection in high-dose IL-2 immunotherapy.

IFN-Gamma is Central to Both Immunogenic and Tolerogenic Properties of Dendritic Cells After IL-12 and GM-CSF Microsphere Treatment

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A single intra-tumoral injection of IL-12 and GM-CSF microspheres results in tumor regression and initiation of an anti-tumor immune response. Activation of NK cells and CD8+ T-cells along

with a decrease in T regulatory cells, after treatment have been observed. The anti-tumor effects of GM-CSF and IL-12 microsphere treatment have been shown to be dependent on IFN-γ. However, further studies demonstrated that the immune response was transient and that the T regulatory cells rebounded rapidly. Recent data from our laboratory suggested that indoleamine 2, 3-dioxygenase (IDO), an IFN-γ-inducible immune-suppressive enzyme, may play a role in the post-therapy T regulatory cell resurgence. Since dendritic cells (DCs) are central to both induction of an immune response, and have been shown to be significant producers of IDO, the effect of IL-12 and GM-CSF microsphere treatment on this potent antigen presenting cell was explored. We found that intra-tumoral injection of IL-12 and GM-CSF microsphere resulted in rapid recruitment of DC to tumors with subsequent migration to tumor-draining lymph nodes (TDLN). Post-treatment DCs displayed increased CD86 expression, a pro-inflammatory cytokine profile and effective $CD8^+$ and $CD4^+$ T cell priming in vitro (immunogenic). By Day 7 post-treatment however, the priming ability of these DCs was completely lost (tolerogenic). Further analysis revealed that day 7 DCs expressed high levels of IDO, inhibition of which resulted in the rescue of the ability to prime T cells. GM-CSF and IL-12 mediated induction of immunogenic DCs was completely abrogated in IFN-γ knockout mice, establishing the critical role of this cytokine in post-therapy immune activation. Importantly, TDLN DCs failed to upregulate IDO and IDO-inhibition did not restore priming function to day 7 DCs in IFN-γ knockout mice, revealing that IFN-γ was also responsible for the induction of tolerogenic function in DCs. These results establish the dichotomous role of IFN-γ in the regulation of IL-12-mediated antitumor immunity and identify DC as the primary conduit that mediate these effects. Furthermore, these data support the hypothesis that blocking IDO in therapeutic regimen designed to induce TH1 responses may prove useful by extending the window of T cell priming and activation.

F19 Labeling of Human α-Type-1-Polarized Dendritic Cells and Standard Dendritic Cells for In Vivo Trafficking by Fluorine MRI

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Human dendritic cells (DC) have great clinical promise as an immunotherapeutic intervention against cancer. A question remains to the dispersion and persistence of DC following administration in assessing therapeutic potency. Here we utilize a perfluoropolyether (PFPE) magnetic resonance imaging (MRI) tracer agent that aims to address these questions in the assessment of α-type-1-polarized dendritic cells (αDC1). αDC1 producing high levels of interleukin-12p70 with putative strong lymph node homing capabilities, are currently being evaluated clinically for anti-cancer activity. 19F MRI tracking is an attractive approach to use for clinical DC tracking because administration of a fluorine label, presents a non-invasive, highly specific image and paired with proton MRI provides clear and quantitative cell localization within anatomic context. Previously, standard DC were evaluated as a "proof of principle" assay in a murine xenograft model to track the migration of human sDC in-vivo by 19F MRI. Mature DC but not immature DC were observed to migrate into the region of draining lymph nodes 18 hours post injection by MRI. Single cell lymph node preparations further determined the presence of DC by flow cytometry. Standard DC were also compared to αDC1 in both function and phenotype, following cellular labeling with PFPE. Standard DC and αDC1 had similar labeling efficiencies with PFPE, which had no effect on the DC's ability to stimulate T cell expansion, produce IL-12p70, process antigen or express co-stimulatory and polarizing factors. These data support the clinical translation of PFPE to safely track and quantify the administration and migration of human αDC1 by non-invasive 19F MRI for cancer patients.

Polarized Dendritic Cells in the Immunotherapy of Established Cancer: Roles of Signal 3 and Signal 4

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Cancer vaccines have been shown capable of prolonging survival of cancer patients, but their ability to induce regression of established tumors remains low. The use of ex-vivo-generated dendritic cells (DCs) helps to sidestep the dysfunction of endogenous DCs in patients with advanced cancer and to deliver the key signals needed for effective anti-tumor responses. Recently, we and others have shown that different DC populations can deliver specialized "signal 3" regulating the acquisition of desirable effector functions by T cells, and an additional "signal 4" that regulates T cell homing properties. Moreover, ex-vivo instruction of DCs can be used to preferentially activate CTLs, Th1- and NK cells, while limiting the undesirable activation of Treg cells. Type-1-polarized DCs (DC1) are characterized by strongly-enhanced, rather than "exhausted", ability to produce IL-12p70 and other CTL-, Th1-, and NK cell-activating factors. A single round of in vitro sensitization with DC1s loaded with tumor-related antigens induces 40 to 70-fold higher numbers of functional CTLs against multiple tumor-related antigens and multiple types of cancer cells (melanoma, glioma, CLL, CTCL, prostate, colorectal, endometrial and ovarian cancers), when compared with immature DCs and nonpolarized mature DCs. DC1s are particularly effective in inducing effector functions in $CD8^+$ T cells, NK cells, and Th1-type $CD4^+$ Th cells (delivery of "signal 3"). They also induce a switch in the expression of chemokine receptors on naïve T cells (delivery of "signal 4"), promoting T cell responsiveness to tumor-produced chemokines. DC1-based vaccines are being currently evaluated in phase I/II clinical trials for patients with different forms of advanced cancer.

Autologous Whole-Tumor Antigen Combinatorial Immunotherapy for Recurrent Ovarian Cancer

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Background: Despite surgical and chemotherapeutic advances, the death rate from ovarian cancer has not changed over the past two decades, warranting the investigation of novel therapeutic strategies. We report the pilot application of combinatorial immunotherapy comprising dendritic cell (DC)-based autologous whole tumor antigen vaccination in combination with antiangiogenesis therapy in patients with recurrent ovarian cancer.

Method: Six patients with recurrent progressive stage III and IV ovarian cancer with available tumor lysate from secondary debulking surgery underwent priming with intravenous bevacizumab and oral metronomic cyclophosphamide (bev/cy × 2 doses), followed by vaccination with DCVax-L, an autologous DC preparation pulsed with autologous tumor lysate (5 to 10 × 10e6 DC per dose, 3 doses) plus bevacizumab (2 doses). Feasibility, safety, and biological and clinical efficacy were evaluated.

Results: Therapy was feasible and well tolerated in all six subjects. Following bevacizumab and oral metronomic cyclophosphamide, vaccination with DCVax-L produced few grade 1 toxicities and elicited tumor-specific T cell responses in four of the six patients. Two of these four patients experienced partial objective clinical

response after completion of vaccine while the remaining two demonstrated stable disease. Among the former, one subject progressed through bev/cy, but had objective response to DCVax-L. Humoral responses were elicited in the 2 patients that experienced objective response, and an HLA-A0201-restricted, Her2-specific T cell response was documented following vaccination in one patient. The over all clinical benefit achieved in this study is 60% (2PR, 2SD and 2PD).
Interpretation: Our results suggest the use of combinatorial cellular immunotherapy comprising bev/cy with DC vaccination with whole tumor antigen for the treatment of patients with recurrent ovarian cancer was well tolerated and warrants further investigation.

Targeting Soluble Tumor Associated Antigens CEA and HER2 to Exosomes Enhances Antigen Specific Immune Reponses

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Patients with cancer may have detectable levels of tumor associated antigens (TAAs) in their circulation, but usually have minimal adaptive immune responses to those antigens. The immunogenicity of soluble protein differs from protein associated with membrane vesicles (exosomes), and this difference may explain the minimal immune response directed against circulating self or TAAs. Adaptive immunity to model TAAs has been enhanced in experimental animal models by directing antigen expression into exosomes. We sought to enhance the immunogenicity of common circulating TAAs by generating recombinant adenoviral vectors expressing the TAA coupled to the factor VIII-like C1C2 domain of milk fat globule epidermal growth factor-factor VIII (MFG-E8)/lactadherin which targets them to exosomes. We created novel adenoviral vectors expressing one of two non-mutated TAAs, including two often found in the circulation of cancer patients, carcinoembryonic antigen (CEA), and the extracellular domain (ECD) of HER2. We compared these novel vectors to vectors expressing the nondirected antigens in animal models to determine if exosomal targeting enhanced immunogenicity. We saw robust improvement in antigen specific immune responses to each of these antigens and conclude that the mode of secretion can influence the immunogenicity of TAAs expressed by viral vectors. This finding may explain their lack of immunogenicity of circulating TAAs, and may be used to enhance future antitumor vaccination protocols.

Comparative Analysis of Cytotoxic T Lymphocyte Cell Response of Dendritic Cells Loaded with Hepatocellular Carcinoma -Derived RNA or Cell Lysate

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The choice of the tumor antigen preparation used for dendritic cells (DCs) loading is important for optimizing DC vaccines. In the present study, we compared DCs pulsed with hepatocellular carcinoma (HCC) total RNA or cell lysates for their capacity to activate T cells. We showed here that HCC total RNA pulsed-DCs induced effector T lymphocyte responses which showed higher killing HCC cell lines ability, as well as higher frequency of IFN-γ production of CD4+ and CD8+ T cells when compared with lysate pulsed-DCs. Both of RNA and lysate loading did not influence the changes of mature DC phenotype and the capacity of inducing T cells proliferation. However, HCC lysate loading significantly inhibited the production of inflammatory cytokines IL-12p70, IFN-γ and enhanced the secretion of anti-inflammatory cytokines IL-10 of mature DCs. Our results indicated that DCs loaded with HCC RNA are superior to that loaded with lysate in priming anti-HCC CTL response, suggesting that total RNA may be a better choice for DCs-based HCC immunotherapy.

A Peptide Derived from EBV-gH Glycoprotein That Reproduces Several Inhibitory Effects of EBV on Monocyte Derived Dendritic Cells

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By interacting with B cells, epithelial cells and monocytes, Epstein-Barr virus (EBV) has been implicated in developing several malignant tumors such as nasopharyngeal carcinoma, Burkitt's lymphoma and the X-associated lymphoproliferative disease. EBV interact with monocyte-derived dendritic cells (MoDCs) mainly through EBV proteins gp350/220 and gp42/gH/gp25 (gp42/gH/gL) complex which binds to CD21 (CR2), MHC Class II and the integrins avb6 and avb8. EBV produces in MoDCs different effects including inhibition of development, apoptosis and induction of cytokines that both stimulate and inhibit activation of Th1 CD4+ T cells (IL-12 and IL-10/IL-6). The glycoprotein gp85 also named EBV-gH protein has a role in the binding of EBV to MoDCs through peptides 11435 (181TYKRVTEKGDEHVLSLVFGK200) and 11438 (241YFVPNLKDMFSRAVTMTAAS260). Here we describe that an analogue peptide from 11438 with a stable alpha-helix (herein named P33210) has similar effects on MoDCs as EBV does. Cells cultured with this peptide remarkably displayed a delay in the MoDCs development evidenced by induction of apoptosis, sustained CD14 expression and impaired expression of DC-SIGN, CD83 and HLA-DR. Interestingly, in MoDCs from several normal donors, P33210 induced high levels of IL-12p70 synthesis in complete absence of IL-10 that persisted longer than that elicited by EBV. Altogether, these results suggest that peptide P33210 may be a tool to dissect activation pathways leading to apoptosis/IL-12 production and/or inhibition of MoDCs maturation induced by EBV.

Induction of Systemic and Therapeutic Antitumor Immunity Using Intratumoral Injection of Bone-Marrow Derived Dendritic Cells Genetically Modified to Express IL-23

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Purpose: We have reported that the systemic administration of IL-23 induces potent antitumor immunity primarily mediated when the Th1-type response is fully promoted in the presence of endogenously expressed IL-12. In this study, we investigated whether bone marrow- derived dendritic cells (BM-DCs) adenovirally transduced with genes encoding murine IL-23 have therapeutic benefits for antitumor immunotherapy.
Experiment Design and Results: We made RDG fiber-mutant adenovirus (Ad) vectors encoding IL-23 or EGFP. The MCA205 fibrosarcoma was intradermally inoculated to C57BL/6, and the mice were intratumorally injected with BM-DCs transduced with Ax3CAmIL23/RGD (Ad-IL-23-DCs) on day 8. The tumors of mice treated with AD-IL-23-DCs resulted in significant growth suppression when compared to that with BM-DCs transduced Ad-EGFP-F/RGD (Ad-EGFP-DCs). Ad-IL-23-DCs treatment induced MCA-205-specific and potent CTL responses in draining lymph node. Furthermore, The NK activity also increased in splenocytes at levels greater than those of Ad-EGFP-DCs. In addition, The significant induction of IFN-γ and IL-17 and decrease of Foxp3+CD4+Tregs in TIL were strongly suggested in the mice injected with Ad-IL-23-DCs when compared with those of Ad-EGFP-DCs.
Conclusion: This strategy designed to deliver genetically modified DCs to tumor sites is associated with systemic and therapeutic antitumor immunity and could be an alternative approach to those

using delivery of DCs loaded with defined tumor antigens. These results support the clinical development of IL-23 gene-modified DCs in patients with cancer.

High-Throughput Analysis of Plasmacytoid Dendritic Cells Reveals Differential Responses to Discrete Viral Entities

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Plasmacytoid dendritic cells (pDC) are key components of the innate immune system capable of synthesizing and rapidly releasing vast amounts of type 1 interferons (IFNs), particularly IFNα. Interaction between activated pDC and T cells in both the lymph nodes and the periphery, as well as the involvement of IFNα in the T-cell mediated control and/or therapy-induced rejection of various viral infections and cancers, makes pDC an attractive but still elusive target for immunotherapy.

Here we investigated whether pDC, often regarded as mere IFN sources for the immune system, are able to discriminate between various functionally discrete stimuli, and to what extent this reflects differences in pDC responses other than IFNα release. To examine the ability and scope of pDC to differentially respond to various doses of intact and infectious HIV, HCV, and H1N1 viruses, whole genome gene expression analysis, ELISA, and flow cytometry, were applied to interrogate pDC responses at the transcriptional and protein level.

Our data demonstrates that pDC differentially respond to various viral stimuli with significant changes in gene expression including those involved in pDC activation, migration, viral endocytosis, survival or apoptosis, CC and CXC chemokine secretion. In some cases, these genes were induced even at levels comparable to that of IFNα. Interestingly, we also found that depending on the viral entity and viral dose, induction of IFNα gene expression and the actual release of IFNα are not necessarily connected. In addition, our data suggests that under certain circumstances, the same virus can support pDC survival or induce apoptosis.

Conclusion: A differential pDC response to different viral stimuli points to the possibility that live viruses or viral components could be exploited to modulate pDC behavior, especially in regards to IFNα and chemokine release in support of biological therapies to treat various cancers. In addition, the distinct pDC responses observed may provide insight into the varying clinical outcomes, including propensity for oncogenesis, resulting from exposure to the three different pathogenic viruses studied here.

Clinically-Compatible 19F NMR Tracking of DC Vaccines In Vivo

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Dendritic cell (DC) vaccines are a valuable tool used in cancer immunotherapies to reset the patient's immune system for enhanced anti-tumor activity. However, the effectiveness of therapeutic vaccines remains limited. The elimination of the antigen-carrying DC vaccines by pre-existing tumor-specific CTLs have been shown to limit the effectiveness of cancer vaccines in the therapeutic settings. While luminescence-based imaging methods can be used to non-invasively and semi-quantitatively evaluate the targeted delivery, migration and persistence of DC vaccines in mice and other small animals, to date there are few robust ways to perform similar studies in humans. Cell tracking of perfluorocarbon-labeled cells by 19F magnetic resonance imaging (MRI) provides a highly-specific signal to quantitatively assess in vivo migration and homing which may be used in clinical studies. CS-1000 is a self-delivering, perfluoropolyether (PFPE) emulsion designed for optimal MRI detection. Here, we tested for the ability to safely and effectively label a novel murine DC vaccine, while not affecting the DCs' maturation status, IL12 production, lymph node-migratory capacity, and their ability to generate type-1 immune responses. In order to determine where the vaccine-carrying DCs are killed by the CTLs, we monitored the migration of DCs from the site of injection to the draining lymph nodes (dLN) using a semi-quantitative bioluminescence-based imaging and the more clinically-relevant and quantitative 19F MRI system. We have determined that PFPE labeling has no adverse side effects on murine DC function. Additionally, these two imaging techniques allowed us to measure the number of DCs at the site of injection (footpad) and dLN which enabled us to determine that vaccine-carrying DCs, when injected into mice with vaccine-specific CTLs, are eliminated at the site of injection without reaching the dLN. These studies verify that the novel CS-1000 can be used to safely monitor vaccine-carrying DCs in vivo.

Identification of Four Conventional Dendritic Cell Subsets in Human Lymph Nodes: Phenotypic and Functional Analysis

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The extensive knowledge which has been gathered over the last decades on the phenotype, localization and specialized functions of Dendritic cell (DC) subsets in secondary lymphoid organs of mice, has unfortunately not led to effective in vivo DC-targeted immunotherapy strategies in humans. In order to take DC-targeted immunotherapy to the desired next level, more insight needs to be gained in the phenotype and functionality of DC subsets in human lymphoid tissue. Here, we report on the identification of four human conventional DC (cDC) subsets in skin-draining human lymph node (LN) material from early-stage melanoma patients. Two CD1a+ subsets were discerned, which were either CD11c_int DC-SIGN- Langerin+ or CD1a+ CD11c_hi DC-SIGN+ Langerin ± (based on intracellular Langerin and DC-SIGN staining). Phenotypically these subsets closely resembled skin-emigrated Langerhans cells and dermal DC, respectively. The other two CD1a-CD11c_hi subsets, which we consider to be more likely blood-derived LN-resident DC, were characterized as CD14- BDCA3+ CD103- and $CD14^+$ BDCA3_int CD103+. The four subsets displayed a differential activation status based on the expression of various co-stimulatory/inhibitory molecules (CD40, CD80, CD86, CD83, B7H1, B7H4). Side-by-side analysis of the FACS-sorted subsets further revealed differences in allogeneic T cell stimulatory capacity, cytokine release profiles, and Thelper-skewing ability. This report is the first to present data from extensive flowcytometric and functional analyses of cDC subsets in human LN, and provides a deeper understanding of DC in human LN as well as new leads for the development of human DC-targeted immunotherapies.

Therapeutic Vaccination With Autologous mRNA Electroporated Dendritic Cells (DC) in Patients With Advanced Melanoma

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Background: mRNA electroporated autologous DC are under evaluation as therapeutic cancer vaccines. Potential advantages are the presentation of all tumor antigen confined epitopes by the patient's own HLA-molecules and the improved immunostimulatory

capacity of the DC-formula. Combination of a therapeutic vaccine with cytokine therapy (IFN-a2b or IL-2) results in a synergistic anti-tumor effect.
Methods: Immature DC (derived from peripheral blood monocytes obtained by leucapheresis and cultured for 6 days in IL-4/GM-CSF supplemented medium) were electroporated with synthetic mRNA encoding a fusion protein between MAGE-A1, -A3, -C2, Tyrosinase, MelanA/MART-1 or gp100, and DC-LAMP, and poly-I:poly-C12U or mRNA encoding TLR-4, CD70 and CD40L (TriMix). DC (12.5 10E6 DC/antigen) were administered by 4 to 6 ID-injections q2w, and q8w thereafter. IFN-a2b (5 MIU TIW) was initiated at progression, concomitant or following the 4th vaccine, respectively in cohort 1, 2, 3, and 4. Immune monitoring was performed by skin biopsy of delayed type IV hypersensitivity (DTH) reactions.
Results: 70 melanoma pts were recruited between 06/′05 and 06/′09: 44 Male/26 Female; med age: 46 years (27 to 75); AJCC stage III: 30, IV-M1a: 8, -M1b: 6, -M1c: 26; WHO-PS 0: 46, 1: 19, 2: 5. A total of 466 DC-vaccines were administered (median/patient: 6, range 2 to 18). Vaccine related AE's included: gr2 injection site reactions: all patients; gr2 fever/lethargy: 3 patients. Vaccinal-antigen specific DTH infiltrating lymphocytes: 0/6 patients tested at vaccine initiation and in 12/21 (57.1%) pts after the 4th vaccine. After a mFU of 30 months, the mRFS for pts without evaluable disease (n = 30) is 23 months (95% CI: 11-34); 3 patients have died, mOS has not been reached. The tumor response among 40 patients with ED at baseline: 1 PR + 14 SD [disease control rate (DCR): 38%] according to RECIST; 2 CR + 2 PR, and 14 SD (DCR: 46%) according to immune-related response criteria (irRC). The 6-mth PFS (32%) and 1-year OS rate (57%) in patients with ED at baseline compares favorably with historical controls. Baseline WHO-PS was identified as an independent covariable for PFS and OS, -CRP for PFS, and -LDH for OS. In a landmark survival-analysis from week 8, DCR by irRC was the strongest independent covariable for superior PFS and OS ($P < 0.001$).
Conclusions: Therapeutic vaccination with autologous mRNA electroporated DC combined with IFN-a2b is feasible, safe, immunogenic and has anti-tumor activity in patients with advanced melanoma. Encouraging survival was observed in this single-arm study and DCR by irRC was strongly correlated with superior survival.

IMMUNE CELL TRAFFICKING TO TUMOR MICROENVIRONMENT

Signatures of Immune Function Genes Associated with Recurrence-Free Survival in Breast Cancer Patients

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We have previously identified signatures of immune function genes associated with tumor rejection, tumor recurrence or tumor progression in FVBN202 transgenic mouse model of neu positive breast carcinoma. In the present study we performed microarray analysis, real-time RT-PCR and immunohistochemistry (IHC) of tumor specimens from breast cancer patients who had either 3 to 7 years relapse-free survival or developed tumor relapse within 1 to 3 years after the initial treatment. Unsupervised analysis showed differential clustering of genes in the tumor specimens of patients with tumor relapse versus relapse-free survival. We determined that network of genes involved in B cell responses, allograft rejection and autoimmune reaction, antigen presentation, and cross talk between adaptive and innate immune responses were exclusively upregulated in patients with relapse-free survival. Interestingly, genes involved in T cell apoptosis, CTLA4 signaling and production of NO and reactive oxygen species were also upregulated in the tumor specimens of patients with relapse-free survival. These data suggest lack of immunological tolerance in breast cancer patients as well as distinct signatures of immune function genes that can be used as prognostic biomarkers for breast cancer patients at the time of diagnosis.Grant support: NIH R01 CA104757 (M. H. Manjili) and MCV Foundation Support-646161 (M. H. Manjili).

NGR-TNF, a Selective Vessel-Targeting Agent, Increases the Therapeutic Potential of Chemo-Immunotherapy

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Novel therapeutic strategies for cancer implement the combination of active and/or adoptive immunotherapies with chemotherapy (chemo-immunotherapy). Indeed, the immune system can target and eradicate small tumor masses, or even single neoplastic cells, but usually fails against bulky tumors. Chemotherapy can reduce the tumor mass, favor the induction of specific immune responses and increase the effector lymphocyte: tumor cell ratio. Unfortunately, the abnormal tumor vasculature and the altered composition of stromal components may significantly impair the penetration of drug and/or effector T lymphocytes into neoplastic tissues, therefore limiting the therapeutic potential of chemo-immunotherapy. One possible strategy to overcome this anatomical and functional obstacle would be to selectively deliver tumor necrosis factor alpha (TNF), a cytokine that can transiently alter the endothelial barrier function, to the tumor vessels.
NGR-TNF is a novel vascular targeting agent currently tested in phase II and III studies in patients with solid tumors. This drug consists of CNGRCG, a peptide capable to home to tumor blood vessels, fused to TNF. In the B16 mouse melanoma and other preclinical models the pre-treatment with picograms of NGR-TNF increases vessel permeability and favors the penetration of chemotherapeutic drugs.
We have found that, at variance with TNF, pre-treatment with NGR-TNF favored the induction of leukocyte adhesion molecules on the melanoma-associated endothelium and the penetration into the tumor mass also of antigen-specific cytotoxic T lymphocytes (CTL) induced either by vaccination or adoptively transferred. In both experimental settings, endogenous and adoptively transferred CTL maintained their effector functions for several days after NGR-TNF treatment, and this phenomenon correlated with a prolonged and statistically significant animal survival.
The therapeutic effect of the combined treatment was amplified by the addition of chemotherapy.Hence, NGR-TNF favors penetration of both drugs and effector T lymphocytes into the tumor mass, and acts in synergy with active and adoptive immunotherapy against melanoma.

Immunohistological Characterization of Tumor Infiltrating Immune Cells in Metastatic Melanomas

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Immune therapy of melanoma relies on the ability of T cells to traffic to the metastatic melanoma microenvironment (MME). The MME contain variable numbers of immune cells, and the intensity of the immune cell infiltrate is believed to correlate with clinical outcome. However, T cell and other immune cells may either promote or limit tumor progression, and systematic characterization of immune cells infiltrating the MME is incomplete. In this study, we investigated the immunophenotype of tumor infiltrating immune cells in 189 metastatic melanomas, by immunohistochemical analysis of a tissue microarray (TMA) and correlation with clinicopathological parameters and clinical outcome. The TMA included LN metastases (46%), Skin metastases (50%), and small bowel metastases (4%). Total CD45+ cells ranged from 0 to 2823

per mm^2 (median 142). Based on median numbers, CD3 T cells accounted for 78%, including CD8 (64%) and CD4 T cells (14%). Macrophage lineage cells (CD163) accounted for 17%, with fewer B cells (3%) and plasma cells (CD138, 2%). Rarely detected were NK cells (CD56), and mature dendritic cells (DC-LAMP). Nuclear staining for FoxP3 was detected on 6% of immune cells. PD1 was detected on less than 2% of cells (median). Numbers of CD8 T cells per mm^2 correlated with survival ($P = 0.0001$), as did numbers of B cells (CD20+, $P < 0.002$), overall immune cells (CD45, $P = 0.002$), T cells (CD3, $P < 0.001$), and plasma cells (CD138, $P = 0.02$). Numbers of macrophages (CD163) did not correlate with survival ($P = 0.91$), nor did NK cells (CD56, $P = 0.64$), mature DC (DC-LAMP, $P = 0.22$), CD4 T cells ($P = 0.11$), or PD1 ($P = 0.71$) or FoxP3 expressing cells ($P = 0.26$). The low frequencies of mature DC suggest that optimal presentation of tumor antigens in the MME may be uncommon; and the low frequencies of NK cells suggest that innate immune mechanisms may have low activity in established melanoma metastases. High proportions of macrophage-lineage (CD163) cells may include MDSC. These findings suggest also that immunophenotypical characteristics of tumor-infiltrating immune cells have prognostic relevance for metastatic melanomas. Importantly, frequencies of immune cells infiltrating tumor differ widely among patients and suggest differential recruitment of immune cell populations, likely representing differences in homing receptors ligands. These differences may impact on the ability of vaccine-induced or adoptively transferred cells to mediate control of metastatic melanoma.

Immunotype of Tumor Infiltrating Immune Cells and its Correlation with Clinical Outcome in Metastatic Melanoma

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Lymphocytes and other immune cells infiltrating melanoma metastases may be associated with an improved prognosis. We have observed that some melanomas have lymphocytic infiltrates that are limited to the perivascular spaces, and we have hypothesized that differentiation of this presentation from more diffuse infiltration may have both biologic and prognostic significance. We have thus systematically evaluated 189 melanoma metastases on H&E sections and have evaluated immune cells (by CD45 stains) and vascular endothelium (by CD34 stains) on a tissue microarray (TMA) containing these tumors. We identified 3 histologic patterns of immune cell infiltration, termed immunotypes: Immunotype A with no immune cell infiltrate; Immunotype B with immune cells cuffing intratumoral blood vessels but not infiltrating among melanoma cells distant from the vessels; Immunotype C with diffuse intratumoral immune cells. These represented 28%, 63%, and 9% of the metastases, respectively. There was no correlation between immunotype and patient age, gender and stage. Survival was best for Immunotype C and worst for Immunotype A ($P = 0.0475$, log-rank test). These findings suggest that the pattern of immune cell infiltration is an important factor in patient survival and may represent a potential prognostic parameter to be reported in melanomas. The cellular composition of the infiltrates differed among immunotypes, with increased B cells (CD20: 22% vs. 4%) and decreased macrophages/histiocytes (CD163: 8% vs. 14%) in Immunotype C tumors compared to Immunotype B tumors. Immunotypes may be useful for categorizing melanoma metastases based on whether homing receptor/ligand interactions in the MME do not (Immunotype A) or do (Immunotype B) favor homing of lymphocytes to the metastases, and whether they receptor/ligand interactions also favor lymphocyte migration from the perivascular space to infiltrate among melanoma cells (Immunotype C). The association of Immunotype C with the best clinical outcomes suggests that these differences are both biologically and clinically significant and may be useful in considering patients and their tumors for immunologic or other therapies.

Trafficking of Positive and Negative Regulatory Immune Cells into the Tumor Microenvironment

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Recent evidence from analysis of human melanoma metastases has suggested that at least two broad categories of tumor microenvironment can be identified with distinct mechanisms of immune escape. One subset of tumors has characteristics of inflammation which includes the presence of multiple immune cell types including dendritic cells and CD8+ effector T cells. These tumors also have signs of innate immune activation and a type I IFN signature. However, the presence of multiple defined negative regulatory mechanisms (Tregs, IDO, PD-L1, and T cell anergy) likely explains tumor resistance to immune destruction. The second subset of tumors is bland, lacks signs of inflammation, and does not contain intratumoral T cells. These tumors are also more vascular, and show evidence of activation of additional oncogenic pathways at the level of the tumor cells. Therefore, lack of effective immune cell recruitment in the tumor microenvironment likely explains tumor escape in these instances. Therefore, understanding the regulation of immune cell trafficking into the metastatic melanoma tumor microenvironment is a critical question that could enable new therapeutic strategies to support the effector phase of the anti-tumor immune response. In murine models, we have found that host type I IFN signals are critical for recruitment of the CD8α+ DC subset into the tumor site, and that this is necessary for endogenous priming of CD8+ T cells against tumor antigens. A set of 6 chemokines has been identified that appear to contribute to recruitment of CD8+ effector T cells into the tumor microenvironment. On the other side of the equation, we have found that CCL22 produced by activated CD8+ T cells contributes strongly to intratumoral recruitment of CD4+CD25+FoxP3+ Tregs. Finally, preliminary data have suggested that disruption of specific angiogenic factors within the tumor site can markedly augment T cell accumulation there. Together, these studies have highlighted specific molecular pathways that are amenable to manipulation to improve upon trafficking of desired immune cell subsets in the target tissue of metastatic tumor deposits.

Effector/Memory Regulatory T Cells and Their Role in the Tumor Microenvironment

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T cell responses are observed in cancer patients who have been treated with antigen-specific vaccines. However, the majority of responses are often weak and ineffective at controlling tumor growth. This may be due to an ineffective vaccine approach. However, in many cases mechanisms of T cell tolerance to specific tumor antigens are at play. Understanding these mechanisms in the context of tumor antigens is critical for the development of interventions that can reverse the tolerant state and allow these T cells to more effectively respond to tumors. We have described the existence of immune tolerance in the HER-2/neu transgenic (neu-N) mouse model of breast cancer and used these mice to understand the mechanisms that suppress high avidity antigen-specific CD8+ T cells. We previously reported that CD8+ T cells specific for the immunodominant neu epitope, RNEU420-429, were identified only in Cy plus vaccine treated neu-N mice that rejected tumor challenge, but not in neu-N mice given vaccine only. Furthermore, high avidity RNEU420-429-specific CD8+ T cells were also identified in vaccine treated mice that were first depleted of CD25+ T regulatory cells (Tregs). More recently we have developed T cell receptor (TCR) transgenic high and low avidity mouse colonies that are specific for the same RNEU420-429 epitope. We have used these mice to evaluate differences in tumor-trafficking and function of high versus low avidity cancer antigen-targeted T cells. Adoptive transfer of naïve T cells from these mice into tumor bearing neu-N mice have identified a sub-set of Tregs

that block high avidity T cell trafficking and activation in neu-expressing tumors. The results of these studies will be discussed. In addition, data will be presented evaluating these findings in a neo-adjuvant and adjuvant vaccine study in patients with pancreatic cancer.

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Tumor Elimination By Depovax™ Cancer Vaccine Platform is Accompanied by Reduced Regulatory/Suppressor Cell Infiltration

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A successful cancer vaccine needs to overcome tumor-induced immune suppression while enhancing protective Type1-biased tumor-specific immune responses. We have developed a novel liposome-based vaccine platform called DepoVax™ (DPX) which increases the potency of peptide-based cancer vaccines and elicits a strong cytotoxic T cell response. DPX-0907 is a human DPX based vaccine containing 7 HLA-A2 restricted peptides indicated for breast, ovarian and prostate cancer which has recently entered Phase I clinical trials. DPX-0907 has shown effective immune induction in HLA-A2 transgenic mice, even in the presence of tumor-induced suppressor cells and cytokines. To investigate the effect DPX vaccines have on the regulatory cell population, we examined the induction and distribution of CD4+ CD25+Foxp3+ (Treg) lymphocytes, immune-suppressive cytokine-secreting Tr1 cells and myeloid derived suppressor cells (MDSC) using a HPV16 tumor model. C57BL/6 mice bearing established C3 tumors were immunized with DPX- or emulsion-based vaccines and regulatory/suppressor cells were examined in the blood, spleen and tumor tissue using flow cytometry and fluorescent microscopy. While emulsion-vaccinated mice showed an increase in Treg cells in both spleen and blood, particularly after repeat immunizations, mice treated with DPX vaccine showed no such increase and the levels remained similar to tumor free naïve mice. Similarly, MDSC levels were significantly lower in DPX-treated mice compared to control mice in spleen, blood and also within the tumor tissue. Production of Tr1-type cytokines IL-10 and TGF-b by CD4+ and CD8+ T cells was significantly increased in untreated and mice treated with emulsion vaccine compared to DPX-treated mice. Analysis of tumor infiltrating T cells revealed DPX vaccinated mice had lower levels of Tregs and higher levels of CD8+ T cells. Furthermore, C3 tumor bearing mice vaccinated with a single dose of DPX-based vaccine containing the H-2Db-restricted HPV16E7 (49 to 57) peptide conjugated to the universal T helper peptide exhibited compete tumor regression two weeks later, which was confirmed by MRI scanning. These findings provide insight into the mechanisms of DepoVax™-based cancer vaccines which may prove advantageous for immunotherapy of immune-suppressed cancer patients.

Proportion of Tregs and Th17 Cells in Peripheral Blood of Patients with Gynecologic Cancer

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Objectives: Regulatory T lymphocytes (Tregs) and T helper type 17 (Th17) cells were known to have a reciprocal function in tumor microenvironment. However, the proportions of Tregs and Th17 cells have not been identified in gynecologic cancer. The aim of this study is to investigate and compare the proportions of Tregs and Th17 cells.

Materials and Methods: Peripheral blood samples were obtained from 50 patients treated with gynecologic cancer and 16 healthy donors in Asan Medical Center from July 2009 to October 2009. Blood samples were stimulated for 4 hours with PMA/ionomycin according to the manufacturer's directions (BD) before intracellular cytokine staining. Cell surfaces were stained with anti CD4 Abs and anti CD25Abs. And then, cells were fixed with fix/perm kit and intracellularly stained with anti Foxp3 Abs and anti IL-17a Abs. The phenotype of lymphocytes was analyzed through the flow cytometry.

Results: Mean age was 50.5 years in cancer patients and 34.8 years in healthy individuals. Cancer patients were composed with 16 cervical cancer, 15 ovarian cancer, 16 uterine cancer and 3 another cancer, and they were all at diagnostic states. We observed the proportion of Tregs and Th17 cells in total CD4+ T lymphocytes. Proportion of CD4+CD25high+FoxP3+ Tregs was $0.64 \pm 0.11\%$ in cancer patients and $0.20 \pm 0.06\%$ in healthy individuals($P < 0.05$). Proportion of CD4+ IL-17a+ Th17 cells was $47.54 \pm 3.81\%$ in cancer patients and $72.06 \pm 2.87\%$ in healthy individuals ($P < 0.05$). The ratio of Tregs/Th17 cells was higher in gynecologic cancer patients than in healthy individuals.

Conclusion: The results have revealed high proportion of Tregs and low proportion of Th17 cells in peripheral blood of patients with gynecologic cancer. It was significantly considered to be important information for improving immunotherapy in gynecologic cancer.

Potential of Immunglobulins Developed from the Genome of B Lymphocytes Infiltrating Tumor Microenvironment for Personalized Cancer Immunotherapy

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Background and Objectives: Aberrant glycosilation occurs in all types of human cancers and many glycosil epitopes constitute tumor associated antigens. Uncontrolled growth, metastatic and invasive properties of tumor cells may be a consequence of certain aberrant sialic acid containing glycosphingolipid (ganglioside) expressions. According to our recent findings, the immune cells infiltrating the tumor tissue can produce antibodies that recognise certain types of GD3 gangliosides. Our objectives were to define the specific binding characteristics of anti ganglioside antibodies of tumor infiltrating B cell origin and other sources. We wanted to get more insight into the tumor associated feature of this target antigen and into the anti proliferative and proapoptotic potentials of the specific antibodies.

Materials and Methods: Unique anti GD3 ganglioside single chain Fv antibody fragments were developed and used for labeling. A panel of cancerous and normal cells as well as tissues were tested in immunofluorescene assay and immunohistochemistry. Various breast cancer (MCF-7, SKBR.3, MDA-MB231), melanoma (SK-Mel 28, SK Mel 24, HT-168, HT-199), lung carcinoma (NCI H661) and colorectal carcinoma (LS174T, HT29) cell types were cultured with anti ganglioside antibodies. MTT cell proliferation assay and various apoptosis assays (Annexin V Apoptosis Detection kit, ssDNA ELISA kit, caspase-9 Colorimetric Assay Kit) were performed.
Results: Strong immunohistochemistry and immunofluorescence activity could be measured in 90 precent of the investigated tumor types. However the expression level was different and varied parallel to the proliferation stage. The majority of the normal cells and tissues were negative, or weak positive, with the small number of exceptions of certain tissue types. Anti GD3 ganglioside antibodies could induce apoptosis events at different levels (from 20%-55%) depending on certain cell types.
Conclusions: Immunglobulin variable region gene fragments obtained from B cells in the tumor tissue reveal key tumor-related antigens, such as specific GD3 gangliosides, that have the potential to influence tumor cell progression. An early detection possibility of these unique ganglioside structures are of high diagnostic interest. The special functional characteristics of these key molecules make a therapeutic usage feasible.
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Spatial and Temporal Regulation of CXCR3 Chemokine Production and CD8 T Cell Infiltration in the Metastatic Melanoma Microenvironment

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Effective immune therapy of cancer requires infiltration of the tumor microenvironment (TME) by tumor antigen (TA)-specific $CD8^+$ T cells (T_{CD8}), a concept demonstrated in murine models and underscored by the prognostic significance of infiltrated T_{CD8} in human tumors. Infiltration of T_{CD8} into the TME is regulated, in part, by chemokines, yet the spatial and temporal regulation of chemokine production in the TME remains poorly characterized. We reported that the presence of circulating TA-specific T_{CD8} expressing the chemokine receptor CXCR3 correlated with a survival advantage in patients with resected stage III metastatic melanoma; further, we have observed that T_{CD8} cells infiltrating human metastatic melanoma are predominantly $CXCR3^+$. However, the induction of $CXCR3^+$ TA-specific T_{CD8} has no prognostic significance in patients with established disease. These data suggest that CXCR3 may facilitate T_{CD8}-mediated immune surveillance and eradication of early metastatic lesions, but that CXCR3 is insufficient to mediate infiltration of late-stage tumors. We hypothesized that the differential capacity of $CXCR3^+$ T_{CD8} to mediate anti-tumor efficacy may reflect the chemokine status of the TME. Therefore, we characterized the spatial and temporal regulation of CXCR3-cognate chemokines in the metastatic melanoma microenvironment. In a murine model of metastatic melanoma growing in the lungs, production of CXCR3-cognate chemokines (CXCL9, CXCL10, and CXCL11) was induced in vascular endothelium adjacent to tumor deposits from day 6 to day 13 of tumor growth. Chemokine production was interferon-gamma (IFN-γ)- and natural killer (NK) cell-dependent, and the presence of chemokine correlated with the capacity of TCR transgenic TA-specific T_{CD8} to infiltrate the tumor-bearing tissues in a CXCR3-dependent manner. In late tumors (> day 13), endogenous IFN-γ and CXCR3-cognate chemokines were not detected, and tumors were refractory to infiltration by TA-specific T_{CD8}, regardless of CXCR3 expression. Exogenous IFN-γ induced CXCL9, CXCL10, and CXCL11 production in the late stage TME and restored CXCR3-dependent T_{CD8} infiltration. Dysregulation of CXCR3-cognate chemokine production in the late-stage tumor was mediated by adenosine; specific blockade of adenosine signaling restored IFN-γ and chemokine production and infiltration of $CXCR3^+$ T_{CD8} in the TME. Thus, early-stage and late-stage tumors are differentially susceptible to immune-mediated infiltration and elimination by effector T_{CD8} as a consequence of the temporal dysregulation of IFN-γ and IFN-γ-induced chemokine production.

Tumor Selective Modulation of Chemokine Expression in Colon Cancer Microenvironment: Strategy to Enhance Teff Homing into Tumors

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Background: Local infiltration with CD8+ effector T cells (CTLs) can predict long-term survival of patients with colorectal cancer (CRC), indicating the key role for the ability of CTLs to enter tumor tissues in the effectiveness of immuno-surveillance of cancer. Current study tries to address the role of chemokines in CTL entry into tumors and further analysis whether T cell homing and entry into tumors with no or less CD8+ TILs can be enhanced by modulation of chemokine expression in tumor Micro-environment.
Methods: Expressions of chemokines in both non-cultured or ex vivo cultured tumor biopsies were analyzed by Taqman, ELISA and In-situ hybridisation. NF-κb was analyzed by confocal microscopy. In vitro Chemotaxis assays were done in 24 well Trans-well plates.
Results: Analysis revealed biased expression in ratio of Treg attracting chemokines versus Teff attracting chemokines in CRC tissues as a result of the prostanoids-mediated over-production of Treg-attracting CCL22/MDC and suppression of Teff-attracting CXCL10/IP10 and CCL5/RANTES. Our data indicate that the combination of prostaglandin synthesis inhibitors with IFNα (in selected patients requiring further supplementation with TLR3-ligand) results in a correction of the tumor-associated chemokine profiles. This treatment induced CXCL10, a Teff attracting chemokine to be selectively prominent in the tumor- tissues rather than marginal healthy tissues. Analysis revealed that the increased propensity of tumors to activate NF-κb on treatment is responsible for this selective tumoral expression of CXCL10. In accordance with the differential ability of Teff and Treg cells to respond to different sets of chemokines, we observed that the COX-inhibitor/IFNα-treatment increases the ability of tumors to attract effector-type CD8+ T cells with a concomitant reduction of Treg-attracting function.
Conclusion: Our data suggests that modulation of tumor environment helps to correct the high Treg/Teff bias, default in colorectal tumors and helps to enhance Teff entry into tumors.

Labeling of T Cells and NK Cells with a Clinically-Applicable Perfluorocarbon for Quantitative 19F MRI Tracking in Cancer Patients

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The effectiveness of adoptively transferred lymphocytes in the treatment of cancer is contingent upon their ability to effectively infiltrate tumors. Tracking therapeutic cells to the tumor microenvironment may provide a non-invasive biomarker of effectiveness, and enable optimization of adoptive therapy for cancer. However, the clinical evaluation of the tumor homing capacity of adoptive cellular therapies has been limited by (1) the paucity of effective methods to specifically and non-invasively image cells in

human cancer patients, and (2) an inability to safely and effectively label human T cells and NK cells.
Cell tracking of perfluorocarbon-labeled cells by 19F magnetic resonance imaging (MRI) provides a highly-specific signal to quantitatively assess in vivo migration and persistence. Previously, 19F MRI has been used to observe and measure the accumulation of antigen-specific murine T cells to anatomic sites in vivo. A novel, self-delivering, perfluoropolyether (PFPE) emulsion designed for optimal MRI detection was tested for the ability to safely and effectively label human primary, in vitro-expanded T and NK lymphocytes.
Activated human T lymphocytes and NK cells and were effectively labeled with PFPE, as measured by NMR spectroscopy, with no evidence of apoptosis or cell death. A dual-mode fluorescent PFPE probe revealed dose-dependent labeling by flow cytometry, and localization of the tracer within vesicles in the cytoplasm using confocal microscopy. The ability of labeled T cells to produce IFN-γ and proliferate in response to TCR stimulation indicated no impairment in these effector functions. Similarly, labeled NK cells exhibited equal IFN-γ secretion and cytotoxicity against target cells as their unlabeled counterparts.
These results indicate that both T cells and NK cells can be labeled with PFPE tracer agents for MRI without alterations in functional properties. The clinical translation of this PFPE tracer for 19F MRI may enable the quantification of effective homing and persistence in the tumor microenvironment in cancer patients, significantly advancing the effectiveness of adoptive immunotherapies.

INNATE/ADAPTIVE IMMUNE INTERPLAY IN CANCER

Monocytes Enhance Natural Killer Cell Cytokine Production in Response to Antibody Coated Tumor Cells in the Presence of IL-12

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Our group has shown in vitro, in murine tumor models and in phase I clinical trials that co-stimulation of NK cells via the interleukin-12 receptor (IL-12R) and the FcγRIIIa activates the extracellular signal-regulated kinase (ERK) signaling pathway, which in turn promotes the secretion of interferon-gamma (IFN-γ) thereby promoting potent anti-tumor effects. We hypothesized that NK cell cytokine secretion would be significantly enhanced following simultaneous stimulation of the NKG2D receptor and that monocytes could serve as a source of NKG2D ligands. Co-stimulation of purified NK cells with trastuzumab coated HER2+ SKBR3 breast cancer cells and IL-12 (10 ng/mL) resulted in synergistic production of IFN-γ (> 20,000 pg/mL) as compared to the single conditions (< 3000 pg/mL). Cytokine production in response to antibody (Ab)-coated tumor cells and IL-12 was significantly enhanced in the presence of autologous monocytes (2-3-fold, $P < 0.05$) but not T cells or B cells. Cytokine secretion peaked at 48 hours, was preceded by a 6-fold increase in IFN-γ transcript and was dependent on cell-cell contact as determined by a Transwell assay. NK cell antibody dependent cellular cytotoxicity was also enhanced in the presence of monocytes. Co-stimulated NK cells co-cultured with monocytes (1:1 = 200,000 cells per well) also secreted significantly higher amounts of TNF-α (> 2 fold) and MIP1 alpha (> 4 fold) as compared to un-supplemented NK cells exposed to Ab and IL-12. A dose-response effect was observed with increasing numbers of added monocytes. Pre-treatment of monocytes with LPS and/or IFN-γ led to increased expression of NKG2D ligands (MICA and MICB) and increased the ability of monocytes to act as co-stimulators of NK cell cytokine secretion. The stimulatory effects of MICA/B+ monocytes could be duplicated by the use of a MICA over-expressing cell line (C1R-MICA) but not the parental MICA-negative cell line (2-3.2 fold increase). Incubation of C1R MICA cells with a MICA neutralizing antibody prior to co-culture with NK cells led to a significant reduction in IFN-γ secretion. The stimulatory effects of monocytes were also observed in whole peripheral blood mononuclear cells (PBMC) in that depletion of monocytes from PBMC markedly inhibited the production of IFN-γ by the NK cell compartment whereas supplementation of PBMC with additional monocytes led to dose-dependent increases in cytokine production. These data suggest that stimulation of NK2D by monocyte ligands can enhance the NK cell cytokine response to Ab-coated targets. Enhancement of NK monocyte interactions could increase the efficacy of Ab-based anti-cancer therapies.

Peripheral Blood Lymphocytes Induce Survival and Autophagy in Human Renal and Bladder Carcinoma Cell Lines

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Objectives/Background: Macroautophagy is an important physiologic process in stressed cells as well as being critical for antigen processing and cross-presentation within Class II MHC molecules. Many tumors expressing stress receptors such as MICA/MICB are susceptible to natural killer (NK) cell induced apoptosis, interacting with NKG2D on the cell surface.
Methodology: We first confirmed that human primary NK cells could kill epithelial cancers cell lines-renal RCC4, T-24 bladder carcinomas and others. NK cells co-cultured with RCC4 renal cancer cells killed their targets at high "effector to target ratios" (E:T, 100:1) following 16 hours in the presence of 500 IU/mL IL-2.
Results: NK cells not only kill targets, but may enhance programmed autophagy/cell survival in epithelial tumors. Interestingly, we have observed enhanced autophagy in the spared target cells in "high NK-kill" conditions. Both the fraction and the number of autophagic cells increased (10% to 64%, 4 to 12 cells per field), comparing no PBLs to 100:1 E:T. The effect was robust (ANOVA $P < 1.7 \times 10$ to 17 within experiment), both with and without IL-2. Also, low effector-target ratios enhanced survival of the cell lines compared with controls (no PBLs added).
Significance: We are now investigating the molecular mechanisms and implication of this 'tumor sparing' immune response which may be a critical antecedent to delivery of autophagic tumor antigens to dendritic cells and be involved in early steps of tumorigenesis.

Melanoma and IRFs: A New Classification of Melanoma

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Background-Interferon regulatory factors (IRF) family members are intimately involved in the regulation of innate and adaptive immunity and oncogenesis. To date, nine human IRF genes (IRF-1 to -9) have been identified. Given their crucial role in host immune defense and tumor suppression, a better understanding of IRF signaling pathways could provide new strategies for the therapy not only of infectious diseases and immune disorders, but also of cancer.
Methods-Transcriptional analysis based on Affymetrix Human gene 1.0 ST arrays was performed on 113 metastatic melanoma metastases. IRF and IRF target gene expression was evaluated by BRB array tool and Ingenuity Pathway Analysis (IPA).
Results-Unsupervised analysis of the 9 IRF gene-expression pattern segregated melanoma metastases into four groups characterized by

respective expression of: (a) IRF-1; (b) IRF-5; (c) IRF-4 or (d) no expression of any IRF (IRF-null). The IRF-1 and the IRF-5 expressing clusters included also the co-expression of IRF-2, 4, 8, 9 and IRF-3, 7, 1, 8 respectively.
Class comparison identified genes differentially expressed (Student's T test $P^2 < 0.05$) by each of the IRFs sub-groups compared to the IRF-null.
IPA demonstrated predominant up regulation of genes associated with: (1) IFN signaling, human leukocyte antigen (HLA) class I and class II and antigen processing in the IRF-1 group; (2) IL-2 and Natural Killer Cell Signaling in the IRF-5 group; (3) IL-4, ILK, IL-9 Signaling in the IRF-4 group.
This classification based on IRF expression pattern and correspondent transcriptional patterns were also observed among established melanoma cell lines, suggesting that these phenotypes are an intrinsic characteristic of cancer cells independent of their interaction with the host's microenvironment.
Conclusion: This novel classification of melanomas according to IRF-transcriptional patterns may provide a new perspective for the understanding the natural history of melanoma and its responsiveness to immunotherapy. Present work is addressing this possibility.

Cancer Cell Death in the Tumor Following Radiation Therapy Causes Infiltration of Immune Suppressive Macrophages

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In our research to identify critical checkpoints in effective tumor immunotherapy we have increasing focused on tumor macrophages as a consistent obstacle in effective adaptive immunity. We propose that the interaction between tumor cells and macrophages plays an important role in tumor evasion of immune surveillance. We investigate the role of the endogenous immune response in murine models of hypofractionated radiation therapy in immunocompetent mice. Mice treated with 3 daily doses of 20 Gy focal radiation beginning day 14 following tumor challenge increased median survival in the Panc02 pancreatic adenocarcinoma model from 29 days to 41 days ($P < 0.001$) and in the 4T1 mammary carcinoma model from 35 to 46 days ($P < 0.001$). Significantly fewer clonogenic cancer cells could be isolated from the treated tumor 1 and 7 days post-treatment; however, despite this cytotoxic effect of radiation therapy, aggressive tumor recurrence was observed in all treated animals and began within 7 to 10 days of the last radiation dose. We have demonstrated in multiple models that depletion of CD8 T cells accelerates tumor recurrence, suggesting that adaptive immune responses transiently control residual disease in the period immediately following radiation therapy. To address the mechanisms of failure of adaptive immune control, we examined tumor-infiltrating cells following treatment. We see significant increases in the number of $CD11b^+$ macrophages within the tumor by flow cytometry both one and seven days following treatment and increased $F4/80^+$ macrophages by immunohistology. Using FACS sorting, we purified $CD11b^+Gr1^{lo}MHCII^+$ macrophages from control and irradiated tumors, and examined their phenotype by microarray analysis and western blotting. We demonstrate that following radiation, tumor macrophages increase their alternative (M2) differentiation, including upregulation of arginase I mRNA and protein. We propose that the increased proportion of macrophages and their increased M2 differentiation promotes an environment that suppresses adaptive immune responses and supports tumor recurrence. Our data suggests that radiation therapy promotes a transient proinflammatory response at the tumor site, accompanied by effective endogenous anti-tumor CD8 immune responses. However, rapid onset of inflammatory resolution limits adaptive immune responses and promotes tumor outgrowth. We are currently investigating therapies that limit the suppressive response of tumor macrophages following radiation therapy.

Establishing an Optimal Tumor Tissue Disaggregation Method for Human Infiltrating Immune Cell Characterization

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Tumors develop immune escape mechanisms which promote their survival and growth, which represent a major obstacle to the success of immunotherapy. We have tried to increase knowledge of the intra-tumor immunological environment in human tumors to obtain better understanding of immune tolerance mechanisms. To characterize immune infiltration from human tumor samples in vitro and to perform reliable studies, it is therefore critical to prepare tumor material in a way that will minimally alter their biology and functions. The first step of our study dealt with the extraction of tumor infiltrating immune cells (TIIC) from solid tumors. Literature describes a plethora of protocols to generate cell suspensions. It has however been reported that some homogenization techniques may induce cell biology changes. We thus proposed to compare effects of three disaggregation tissue methods on TIIC biology. We attempted to identify a method of generating single cell suspensions without specifically inducing cell death, loss of cell surface markers, or inhibition of cell proliferation.
To perform these experiments, individual human breast, lung or kidney tumors were first cut into small pieces with a scalpel. Cell suspensions were obtained after treatment with type IA collagenase, a cocktail of type IA collagenase, type IV collagenase and DNase I type II, or by mechanical disaggregation by Medimachine™. Initially, cell mortality was determined by flow cytometry after dead cell staining. Then, loss of cell surface markers was quantified by staining of eight commonly used markers for leukocyte characterization. Finally, effects of these extraction methods on cell proliferation were quantified after CFSE staining, anti-CD3 and IL-2 activation and four day culture. Results presented demonstrate that mechanical disaggregation by Medimachine™ induced a slight increase in cell mortality while maintaining cell surface marker integrity and cell proliferation. Specifically, treatment with enzymes coccktail (collagenases and DNase) provokes a strong decrease in CD4, CD45-RA and CD14, and a decrease of proliferation when compared with Medimachine™. In summary, the Medimachine™ appears to be more efficient in preparing TIIC with minimal cell alteration.

Pathogenic Mast Cell/T Regulatory Cell Cross Talk in Colorectal Cancer

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Colorectal cancer (CRC) is one of the most common fatal malignancies worldwide. Almost 5% of the adult population in the United States will suffer from CRC, and half of the affected individuals will die from this disease. CRC is driven by inflammation and understanding immune mechanisms that regulate inflammation could produce breakthroughs in the treatment of this disease. We have evidence suggesting that inflammation in CRC is regulated through the cross talk between tumor infiltrating mast cells (MC) and T regulatory cells (Treg). This is based on three sets of observations, first showing a causative role for MC in the progression of pre-neoplastic lesions to carcinoma, second demonstrating the inherent potential of Treg to suppress MC, inflammation, and polyp growth, and third our discovery that Treg in mice or patients with progressive cancer are reprogrammed to become pro-inflammatory and stimulate MC. Pro-inflammatory Treg express Foxp3 and are potently T cell suppressive, but have characteristics of TH17 cells, including expression of RORγT and IL17, and are poor IL10 producers. Genetic ablation of RORγT or IL17 or IL23 in bone marrow attenuates polyposis and blocks the pro-inflammatory differentiation of the Treg.

Our observations are consistent with the notion that Treg have an anti-inflammatory and protective role in CRC, however this role is compromised by their interaction with MC in the course of progressive disease. Thus, the cross talk between MC and Treg determines the level of inflammation in CRC. The shift of Treg to a pro-inflammatory phenotype is a turning point in the escalation of cancer-associated inflammation[1,2] (see commentary[3]). Based on these observations we propose that MC and their interaction with Treg are suitable targets for effective therapeutic intervention in CRC.

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Roles of Interleukin-4 Receptor α-chain on Glioma-Infiltrating Monocytes

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Several epidemiological studies have indicated association of single nucleotide polymorphisms and haplotypes in the IL-4Rα gene with altered glioma risk and prognosis. IL-4Rα is expressed on immunosuppressive cells of monocyte lineage and mediates their production of transforming growth factor (TGF)-β in response to IL-4 or IL-13. In this regard, our evaluation of tumor-infiltrating leukocytes in human malignant gliomas (n = 7) revealed that glioma-infiltrating $CD14^+$ monocytes express high levels of IL-4Rα. By contrast, $CD14^+$ monocytes in peripheral blood expressed barely detectable levels of IL-4Rα, suggesting the unique up-regulation of IL-4Rα in the glioma microenvironment. Based on these observations, we sought to address the functional significance of IL-4Rα in a recently developed murine de novo glioma model. We induced de novo gliomas in BALB/c-background mice by intracerebroventricular transfection of oncogenes using the Sleeping Beauty transposon system. IL-4Rα-deficient (Il4rα−/−) mice exhibited significantly prolonged symptom-free survival compared with WT mice; median survival was 89 and 55.5 days, respectively. Consistently, gliomas induced in the WT mice were infiltrated with significantly higher numbers of $CD11b^+Gr1+$ immunosuppressive monocytes than those in the Il4rα−/− mice. We subsequently isolated glioma-infiltrating $CD11b^+Gr1+$ monocytes to address their functions. RT-PCR and ELISA revealed that the monocytes derived from WT mice express significantly higher levels of TGF-β. Since TGF-β is known to inhibit the function of effector T cells, we addressed the significance of T cells in glioma development by inducing gliomas in Rag1-deficient (Rag1−/−) mice, which lack adaptive immune cells including T cells. Rag1−/− mice exhibited significantly shortened survival compared with WT mice. Taken together, these data suggest that IL-4Rα expression on glioma-infiltrating monocytes may promote the immunosuppressive microenvironment of gliomas through a variety of mechanisms including TGF-β production and T cell inhibition, thereby facilitating glioma growth.

Myeloid-derived Suppressor Cells and Decreased Interferon Responsiveness in Tumor-Bearing Mice

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Our group and others have determined that immune effector cells from patients with advanced cancers exhibit reduced activation of IFN induced signaling pathways although the mechanisms underlying this observation have not been delineated. We hypothesized that increases in myeloid-derived suppressor cells (MDSC), which are known to be elevated in the setting of advanced cancers, could interfere with the host immune response to tumors by inhibiting immune cell responsiveness to interferons. The C26 murine adenocarcinoma model was employed to study immune function in advanced malignancy. This model can mimic advanced disease in humans by the development of cancer cachexia which is associated with weight loss, aggressive tumor growth, and elevated levels of IL-6. Splenocytes from tumor-bearing mice exhibited reduced phosphorylation of STAT1 (P-STAT1) on Tyr 701 ($P < 0.0001$) in response to IFN alpha or IFN gamma. This inhibition was seen in CD4+ and CD8+ T cells, as well was CD49b+ NK cells in mice. C26 bearing mice had significantly elevated levels of $GR1+CD11b^+$ MDSC as compared to control mice ($P < 0.0001$). In vitro co culture experiments revealed that MDSC inhibited IFN responsiveness of splenocytes from normal mice. Treatment of C26-bearing mice with gemcitabine or an anti-GR1 antibody led to depletion of MDSC and restored splenocyte IFN responsiveness. Spleens from C26 bearing animals displayed elevated levels of iNOS protein and nitric oxide (NO). In vitro treatment of splenocytes with a nitric oxide donor led to a decreased STAT1 IFN response. The elevation in NO in C26-bearing mice was associated with increased levels of nitration on STAT1. Finally, splenocytes from iNOS knockout mice bearing C26 tumors exhibited a significantly elevated IFN-response as compared to control C26 tumor bearing mice. These data suggest that NO produced by MDSC can lead to reduced interferon responsiveness in immune cells.

Correlation of the Expression of Hepcidin mRNA and the Serum Iron, Ferritin and TIBC Levels in Hepatocellular Carcinoma

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Background: The present study correlated the expression of hepcidin mRNA and the levels of serum iron, ferritin and TIBC in hepatocellular carcinoma (HCC).

Methods: Samples of cancerous and non-cancerous liver tissue were taken from patients with HCC who underwent hepatectomy. Expression of hepcidin mRNA was evaluated by real-time PCR, and compared in tumors differing in their degree of differentiation and number of tumors. Correlations between hepcidin expression and the serum concentration of hepcidin were evaluated, together with the expression of the serum concentration of biochemical markers of iron metabolism.

Results: Hepcidin mRNA expression in non-cancerous and cancerous tissues was 1791.6 (34.3 to 25187.4) and 58.7 (1.9 to 3235.8), respectively ($P < 0.0001$). There were no significant differences in hepcidin expression among tumors differing in their degree of differentiation and number of tumors. The serum concentration of hepcidin did not correlate with hepcidin-mRNA expression. The serum iron level was found to be normal (125.4 ± 21.4 mg/dL). The serum ferritin level was also found to be normal 193.5 (14.0 to 232.9 ng/mL). TIBC was (284.8 ± 28.3 ng/mL). There were significant correlations between the serum levels of hepcidin and iron, hepcidin-25 and ferritin and hepcidin and TIBC.

Conclusion: The serum hepcidin concentration was correlated with the levels of serum iron and ferritin, but not with the level of hepcidin mRNA expression in either cancerous or non-cancerous liver tissue. Hepcidin is produced in patients with HCC, from non-cancerous liver tissue, even though production is inhibited in cancerous tissue. Expression of hepcidin mRNA is constitutively suppressed in cancerous, but not in non-cancerous liver tissue of patients with HCC.

Immunoregulation by Invariant Natural Killer T Cells in a Mouse Model of Metastatic Breast Cancer

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Immunoregulatory and suppressive mechanisms are major obstacles to the success of immunotherapy in cancer patients. Invariant natural killer T (iNKT) cells have been shown to down-regulate the immune response in autoimmune diseases, but to up-regulate anti-tumor immunity when activated by α-galactosylceramide (α-GC). We have previously shown that the combination of radiotherapy (RT) to the primary tumor and CTLA-4 blockade induces a CD8-mediated anti-tumor response inhibiting metastases and extending the survival of mice bearing the poorly immunogenic and highly metastatic 4T1 mammary carcinoma. Surprisingly, the response to treatment was markedly enhanced in the absence of iNKT cells, with 50% of iNKT cells-deficient (iNKT $-/-$) mice versus 0% of WT mice showing complete tumor regression, long-term survival, and resistance to a challenge with 4T1 cells. Activation of iNKT cells by α-GC administration to wild type (WT) mice with established 4T1 tumors did not enhance the response to treatment. In the absence of treatment, tumors grew similarly in WT and iNKT $-/-$ mice. Repeated vaccination with irradiated tumor cells did not induce protective immunity in either WT or iNKT $-/-$ mice, indicating that the tumor is not differentially immunogenic in iNKT $-/-$ mice. However, spontaneous lung metastases were reduced in iNKT $-/-$ as compared to WT mice, and this difference was eliminated by depletion of CD8 T cells, suggesting that development of anti-tumor CD8 T cell responses is enhanced in the absence of iNKT cells. Interestingly, whereas WT and iNKT $-/-$ mice showed a similar systemic and intratumoral increase in myeloid-derived suppressor cells (MDSC), the numbers of intratumoral CD11c+ dendritic cells (DCs) was significantly higher in iNKT $-/-$ mice ($P < 0.001$) Furthermore, even in the absence of treatment, DCs obtained from tumor and tumor-draining lymph nodes of iNKT $-/-$ mice showed increased expression of maturation markers (CD40, CD80, CD86, MHC-II) compared to DC from WT mice. This data suggests that iNKT cells may negatively regulate cross-priming of anti-tumor CD8 T cells at the level of the antigen-presenting cells in this tumor model. Supported by DOD BCRP Postdoctoral Award BC086964 to KP.

Generation of an Innate Immune Microenvironment as a Novel Mechanism for Myotoxins to Potentiate Genetic Cancer Vaccines

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We recently reported that administration of low doses of myotoxins at vaccination sites potentiated antigen-specific T-cell immunity and tumor protection induced by genetic lymphoma idiotype vaccines in mice, an effect which was superior to TLR agonists. In the current study, we found unexpectedly that the mechanism of this potent adjuvant effect was immune-mediated. Myotoxins induced sterile inflammation at vaccination sites, associated with a predominant infiltration of dendritic cells (DC). Inhibition of DC recruitment abrogated the immune stimulation effect of myotoxins, suggesting the requirement for DC. Genetic profiling of myotoxin-treated tissues revealed characteristics of an immune microenvironment with upregulation of chemokines, proinflammatory cytokines, Toll-like receptors (TLR) and their endogenous ligands, and activation of innate immunity. Mechanistic experiments in vivo also elucidated the requirement for genes triggering DC maturation including TLR signaling and CD40. These studies suggest that myotoxins-induced sterile inflammation generates a favorable innate immune microenvironment that promotes multiple stages in the development of adaptive immunity. This novel mechanism of immune potentiation may be exploited for development of adjuvants for genetic vaccines against cancer.

Interferon-β Secretion in the Tumor Microenvironment can Cause Potent Tumor Control Through Host Cells Independently from Adaptive Immunity

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Innate immune recognition of tumors is essential for generating a natural adaptive anti-tumor immune response. We have recently shown that host interferon-β (IFN-β) production is required to generate a primary adaptive immune response against B16-F10 melanoma and other murine transplantable tumors. This fundamental observation generated the hypothesis that provision of exogenous IFN-β in the tumor microenvironment might augment spontaneous adaptive immune responses even further, perhaps to the point of promoting complete tumor rejection. To test this notion, we retrovirally transduced the murine IFN-β cDNA into B16-F10 melanoma cells, which expressed the model SIYRYYGL (SIY) antigen to enable monitoring of T cell dynamics. In an autocrine manner, these cells subsequently secreted the chemokine CXCL10 and upregulated MHC class I surface expression, supporting a positive immunomodulatory effect. Upon implantation into C57BL/6 mice in vivo, IFN-β expressing B16-F10 tumors initially grew but then were potently controlled, even at doses up to 6×10^6 cells. A mixed population of wildtype and IFN-β-expressing B16-F10 melanoma cells was also completely controlled. Moreover, 10-day established B16-F10 tumors were completely rejected after intratumoral injection of IFN-β-expressing B16-F10 melanoma cells, arguing for a potent bystander effect. Expression of the type I interferon receptor (IFNAR1) on host cells was required to mediate this suppression of tumor growth. However, tumor control was not associated with significantly increased T cell responses as measured by IFN-γ ELISPOT or tetramer analysis specific for the SIY antigen, suggesting that improved adaptive immunity might not be the mechanism at work. Strikingly, identical tumor control was observed in Rag2$^{-/-}$ mice, arguing that T cells and B cells were dispensable. In addition, mice depleted of NK cells also demonstrated control of IFN-β-expressing tumors. Interestingly, IFN-β-expressing tumors showed a massive increase in macrophage infiltration in the tumor microenvironment, which may be immune effectors in this setting. Therefore, local IFN-β expression in the tumor microenvironment can mediate strong anti-tumor effects mediated by the host, independently of T, B, or NK cells.

Adenovirus-Engineered Human Dendritic Cells Effectively Recruit Natural Killer Cells via CXCL8/IL-8 and CXCL10/IP-10

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Recombinant adenovirus-engineered dendritic cells (Ad.DC) are an effective modality for inducing anti-viral and anti-cancer T cell immunity. The effectiveness of Ad.DC-based vaccines may depend on the ability of Ad.DC to crosstalk with natural killer (NK) cells and to activate, polarize, and bridge innate and adaptive immunity. Previously we reported that human Ad.DC can efficiently activate NK cells by cell-to-cell contact. In order for this interaction to occur, Ad.DC must be able to effectively attract NK cells. In the present study, we evaluated the ability of Ad.DC to recruit resting NK cells in vitro. We found that Ad.DC effectively recruited both CD56hi CD16- and CD56lo CD16+ NK cell subsets. Resting NK cells consistently expressed CCR7 (CCL19/MIP-3β receptor; both subsets), CXCR1 (CXCL8/IL-8 receptor; CD56lo CD16+ subset) and CXCR3 (CXCL9/MIG and CXCL10/IP-10; both subsets), while Ad.DC secreted high, moderate and minute levels of CXCL8/IL-8, CXCL10/IP-10, and CCL19/MIP-3β, respectively, but no CXCL9/MIG. These findings suggested that CXCL8/IL-8 and CXCL10/IP-10 were the most likely ligands involved in chemotaxis.

Blockade of CXCL8/IL-8 and CXCL10/IP-10 resulted in dramatic and moderate inhibition of NK cell migration, respectively. Additionally, recombinant CXCL8/IL-8 induced specific chemotaxis of CD56lo CD16+, while CXCL10/IP-10 attracted primarily CD56hi CD16- NK cells. Overall, these data show that Ad.DC effectively recruit both major subsets of NK cells, which, coupled with their ability to activate NK cells, makes them potent immune activators of not only adaptive, but also innate immune responses. The study also newly defines CXCL8/IL-8 as one of the crucial mechanisms by which DC recruit NK cells.

Functional Reconstitution of NKT Cells in Cancer and Chronic Viral Diseases

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Natural killer T cells are an innate type of immune cells responsible for first line of anti-cancer and anti-viral defense, through crosstalk with downstream antigen presenting cells, NK cells, and adaptive immune cells. Previous studies indicate that activation of NKT cells in mouse models of human diseases may elicit tumor or virus-specific adaptive immune responses. Alpha-galactosylceramide, a marine sponge-derived agonist ligand for NKT cells, has been evaluated for its efficacy in treating cancer and hepatitis in several clinical trials. In this study, we evaluated the effect of repeated treatment with alpha-galactosylceramide-loaded dendritic cells (αGalCer/DC) on adaptive immunity, in rhesus monkeys infected with SHIV. We found that NKT cells could be expanded in vivo, and were capable of producing anti-viral cytokines. Downstream activation of SHIV-specific CD8 T cells was observed in 4 out of 7 SHIV-infected rhesus monkeys treated with αGalCer/DC. αGalCer/DC treatment was safe, and did not cause viral replication in infected monkeys. Although previous studies suggested that the function of NKT cells is severely impaired in HIV-1 infected human individuals, we found that NKT cells could be expanded from HIV-1 patients with low and medium viral load, but not from advanced AIDS patients. Our results indicate that NKT cells in cancer and chronic viral infections are interesting targets for therapeutic intervention. Activation of NKT cells may induce anti-cancer and anti-viral cytokine release and activation of adaptive immune cells. Our data also encourage development of NKT activating pharmaceuticals targeted at dendritic cells, such as alpha-galactosylceramide conjugated to biodegradable nanoparticles.

In Vivo 6-thioguanine-resistant T Cells from Melanoma Patients Contain T Cells with Melanoma Specificity as well as T Cells with Suppressive Function

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In vivo hypoxanthine-guanine phosphoribosyltransferase (HPRT)-deficient T cells (MT) from melanoma patients are enriched for T cells with in vivo clonal amplifications and can traffic between blood and tumor tissues. The purpose of this study was to determine if in vivo MT from metastatic melanoma patients contained T cells with melanoma specificity as well as T cells with suppressive function (Tregs). MT were obtained by 6-thioguanine (TG) selection of lymphocytes from peripheral blood and from sites of melanoma tumor, and wild-type T cells (WT) were obtained analogously without TG selection. Using a pool of 5 peptide-MHC Class I pentamers for melanoma-associated antigens, a significant enrichment of melanoma-specific T cells in MT compared to WT was demonstrated in 3 of 5 tumor mass cultures ($P < 0.001$); a similar enrichment for MT compared to WT was not seen in 9 peripheral blood cultures (0 of 9). In two independent assays, the MT mass culture from the peripheral blood of an initial melanoma patient significantly suppressed the proliferation of naïve allogeneic CD4+ CD25- T cells (Tresp) to mitogenic stimulation at a 1:1 ratio (18% and 34%, $P = 0.012$ and $P = 0.0001$, respectively), which was ameliorated with dilution. In contrast, WT from that patient showed only weak suppressive potential in only one of the assays (9%, $P = 0.043$). T cell clones from the peripheral blood of the same patient were examined for suppressive function. Two MT clones completely abrogated the Tresp proliferation via a mixed lymphocyte reaction (MLR) at a 1:1 ratio, and this effect was dose dependent. Current experiments will determine whether Tregs can be identified in additional melanoma patients and whether Tregs are enriched in MT compared to WT in the peripheral blood and tumor of patients with metastatic melanoma. We conclude that in vivo MT from melanoma patients contains T cells with melanoma specificity as well as T cells with regulatory activity, and further study of MT in melanoma patients is warranted.

TARGETED THERAPEUTICS AND IMMUNOTHERAPY

Pre-Clinical and Clinical Evaluation of Topical TLR7 Agonist Imiquimod for the Treatment of Breast Cancer

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Background: Toll-like receptor (TLR) agonists are attractive agents for the active immunotherapy of cancer. Local administration of TLR7 activator imiquimod (IMQ) creates an inflammatory environment suitable for tumor antigen cross-presentation and infiltration by effector T cells. Like IMQ, radiotherapy (RT) is a local modality that can alter the tumor microenvironment and enhance tumor immunogenicity, and we have previously shown its ability to synergize with immunotherapy. To test the therapeutic potential of topical IMQ alone or in combination with local RT for the treatment of subcutaneous (s.c.) breast cancer we employed the poorly immunogenic TLR7-negative TSA mouse breast carcinoma model injected into syngeneic immunocompetent mice.

Methods: TSA cells (1×10^5) were injected s.c. into Balb/c mice at the right flank. On day 10 when tumors became palpable, mice were randomly assigned to 4 groups (N = 6 to 10/group): topical IMQ 5% or placebo cream was applied 3 × per week for up to 4 weeks with or without local RT (8 Gy × 3 fractions, days 12, 13 and 14). Treatment response was determined by measuring tumor growth and survival of mice.

Results: Tumor-bearing mice treated with IMQ alone showed delayed tumor progression in comparison with control mice ($P < 0.0001$ on day 25) and increased infiltration by dendritic cells, CD4 and CD8 T cells. RT as single modality also delayed tumor growth; however, neither treatment by itself was able to induce complete tumor regression. When IMQ was given in combination with RT, there was enhanced tumor inhibition ($P < 0.05$), accompanied by markedly increased infiltration of CD8 and CD4 T cells at day 25, and complete tumor regression was observed in

4/6 mice at day 35. Depletion experiments confirmed that the anti-tumor response of IMQ was CD8 T cell mediated. Therefore, mice were injected in both flanks with TSA cells, to assess systemic tumor control. Local treatment with IMQ to one tumor resulted in significant inhibition of a second placebo-treated tumor ($P < 0.01$). Combinatorial local treatment with RT + IMQ to one tumor also resulted in significant inhibition of a second tumor outside of the radiation field, which was potentiated when the latter also received topical IMQ.

Conclusions: Overall, results indicate that synergistic anti-tumor effects are obtained when local RT is administered coincident with TLR activation. A Phase II trial of topical IMQ for the treatment of breast cancer metastatic to skin is currently ongoing, combinatorial approaches are planned after completion of the pre-clinical evaluation.

SCV-07 as an Intervention for Oral Mucositis (OM) in Head and Neck Cancer (HNC) Patients Receiving Chemoradiation (CRT)-Phase II Trial Results

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Background: OM is a significant toxicity of CRT regimens used to treat HNC. This trial was a phase II, randomized, double-blind, dose-ranging, placebo-controlled three-arm study in patients receiving CRT for the treatment of HNC, to assess the safety, tolerability and efficacy of gamma-D-glutamyl-L-tryptophan (SCV-07) as an intervention for ulcerative forms of OM as assessed by WHO OM scale criteria.SCV-07 is a synthetic peptide that is effective (Pouliot et al, 2010) in attenuating radiation-induced OM in a clinically predictive animal model (Alvarez et al, 2003). The effectiveness of SCV-07 in this model may result from SCV-07 immune modulating activities (Tuthill et al, 2009).

Methods: 59 patients with pathologically-confirmed, non-metastatic squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx or larynx were enrolled and treated with CRT consisting of a continuous course of external beam irradiation (IMRT eligible) with a minimum cumulative dose of 50 Gray (Gy) and cisplatin monotherapy administered using standard weekly or tri-weekly dosing regimens (80 to 100 mg/m^2 administered on Days 0, 21 and 42) or weekly (30 to 40 mg/m^2). At least two "at risk" intraoral sites were included in the radiation field and received a cumulative RT dose of at least 50 Gy. Subjects were equally randomized to receive either placebo or one of two doses of SCV-07 (0.02 or 0.1 mg/kg) on each radiation day for up to 7 weeks.

Results: Analysis of adverse events showed that SCV-07 was safe and well tolerated. Regarding efficacy, subjects given the higher dose of SCV-07 demonstrated a 44% decrease in severe OM (WHO grade > 2) compared to placebo at cumulative radiation doses < 40 Gy (18% of 17 subjects on SCV-07, versus 32% of 20 subjects on placebo). At 50 Gy, severe OM incidence was still 30% lower in these subjects compared to placebo (29% vs. 42%). High dose subjects also experienced a delay in onset of ulcerative OM (WHO Grade > 1; $P = 0.085$) and prevention of ulcerative OM in 24% of subjects up to 50 Gy, while all subjects on placebo had ulcerative OM by 35 Gy. Cox regression analysis of time to initial occurrence of ulcerative OM resulted in a 52% decrease in risk for subjects on high dose SCV-07. This group also had 75% fewer unplanned office- and emergency room visits, fewer RT interruptions due to OM, and 14% fewer days when gastrostomy tubes were used vs. placebo.

Conclusions: SCV-07 may be an effective intervention for the development of OM in patients being treated for HNC. Additional dose optimization studies are planned.

Checkpoint Blockade in Tumor Immunotherapy: New Insights and Opportunities

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Over the past several years it has become apparent that the effectiveness of active immunologic strategies for cancer therapy have been limited by cell intrinsic and extrinsic regulatory pathways that act in concert to minimize harm to normal tissues. The prototype of cell intrinsic "checkpoints" whose blockade enhances anti-tumor responses is CTLA-4, which has been extensively studied in a large number of animal models and shown to be quite effective in achieving, either as a single agent or in combination with other agents, complete tumor eradication and long lasting tumor immunity.

Over 4000 patients have been treated with an antibody to human CTLA-4 (Ipilimumab, Medarex and Bristol-Myers Squibb). Significant responses, including complete remissions, have been observed in about 15% of metastatic melanoma patients, with about 40% of patients showing survival benefit. In a recent Phase III trial, Ipilimumab was shown to prolong survival of stage IV metastatic melanoma patients, with 25% alive and ongoing at 4 years. This is the first drug ever to show a survival benefit in metastatic melanoma in a randomized trial. This has led to considerable effort to identify biomarkers that would be useful in determining the impact of CTLA-4 on human immune responses in order to identify changes that might correlate with clinical responses, as well as to address combinatorial strategies that might enhance the effectiveness/frequency of clinical responses.

In both mouse and man, clinical benefit seems to correlate with an increase in the ratio of Teff/Treg cells and with an increase in the proportion of IFNγ producing Teff cells that express high levels of the CD28/CTLA-4 homolog ICOS. In human prostate and melanoma patients clinical responses appear to correlate with pre-existing or induced high titer antibody and polyfunctional CD4 T cells to the cancer testes antigen NY-ESO-1. In a presurgical bladder cancer trial it has been shown that anti-CTLA-4 treatment results in an increase in the ratio of IFNγ producing effector cells that express high levels of the CD28/CTLA-4 homolog ICOS. We have confirmed this in ICOS^{high} CD4 T cells in metastatic melanoma and hormone refractory metastatic prostate cancer. In melanoma, our data suggest that the duration of elevation of ICOS^{high} T cell numbers correlates with favorable clinical outcome. We have begun to explore combinations of anti-CTLA-4 with other agents.

We have found that with proper consideration of dosing and timing, anti-CLTA-4 can synergize with standard chemotherapy, cryoablation, and targeted therapies.

Increased Myeloid Derived Suppressor Cells in Advanced Prostate Cancer

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Prostate cancer is one of the leading causes of death in adult men. Whilst prostatectomy and radiotherapy are potentially curative for organ-confined diseases, there is currently no effective treatment for locally advanced and/or metastatic cancer. As prostate cancer expresses several unique tumor-associated antigens, immunotherapy affords a promising alternative approach to treat this disease. However, although vaccination strategies tested in phase I trials and a randomized phase III vaccine trial in prostate cancer showed encouraging clinical results, they were still far from optimal. One major explanation for this is the fact that many patients with advanced cancer are immune suppressed and are unable to activate tumor-reactive T cells. Recent studies have shown that myeloid-derived suppressor cells are major obstacles to effective cancer immunotherapy as they are potent immune suppressive cells that accumulate to high levels in response to tumor-secreted and pro-inflammatory factors. Whilst there is now considerable evidence

showing the increased levels and immune suppressive ability of these cells in patients with different types of cancer, there is at present no published data investigating MDSCs specifically in prostate cancer. Furthermore, prostate cancer is a particularly good model with which to study MDSCs as the disease has various stages including patients under active surveillance, those with localised disease and those with advanced stage disease. By investigating at what point MDSCs become prevalent in the disease progression allows us to understand when immune suppression becomes important. We have collected peripheral blood samples from prostate cancer patients at each of these stages of the disease and are currently determining the frequencies of MDSCs present. We have already shown a significant increase ($P < 0.0001$) in the percentage of MDSCs in the blood of advanced stage prostate cancer patients (mean: 2.6%, SD: 1.9, range: 0.19% to 7.5%) compared to age-matched male healthy controls (mean: 1.0%, SD: 0.38, range: 0.56% to 1.6%). We will also determine which of the multiple mechanisms that MDSCs use to inhibit antitumor immunity are operative in prostate cancer. A better understanding of the role of MDSCs in prostate cancer is critical in order to optimize strategies that would enhance the therapeutic efficacy of immunologic interventions.

EN2: A Novel Immunotherapeutic Target for Melanoma

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The world wide incidence of melanoma has been constantly increasing during the last years. Whilst surgical excision is effective when primary tumors are thin, at later stages of the disease patients often succumb due to failure of metastasis control. Several studies have now shown the existence of cell-mediated immunity in patients with advanced metastatic melanoma. Thus identifying and targeting clinically relevant antigens for immunotherapy offers a promising alternative strategy to treat metastatic melanoma patients. We have identified one such promising target antigen, the homeobox transcription factor ENGRAILED 2 (EN2). EN2 is specifically involved in patterning the region that gives rise to the cerebellum but more recently has been shown to be a candidate oncogene in breast and prostate cancer. Having performed an immunohistochemical study on a high density malignant melanoma tissue array we have found that 60% and 57% of malignant melanomas and metastatic melanomas respectively express EN2. This is in contrast to no expression of EN2 in normal skin or other normal tissues. We have used a reverse immunology strategy to identify several immunogenic HLA-A2 restricted EN2 epitopes with which we were able to generate EN2-specific CTL responses from the blood of both HLA-A2 positive healthy control donors and melanoma patients. As the maximum immunotherapeutic potential is achieved by antigens that can elicit both a cell-mediated and humoral response, we are currently screening the sera from a large cohort of melanoma patients for IgG autoantibodies to EN2 and comparing this to control sera from healthy age matched donors with no history of cancer. We have preliminary data showing a beneficial effect of EN2 vaccination in a mouse model of melanoma where vaccinated animals developed a much smaller tumor than controls. The fact that these animals also had a positive recall antigen response to the vaccine shows that the tumor outcome may well be immune mediated. We are currently repeating these experiments. We conclude from these data that EN2 is a promising target for melanoma immune therapy.

Exceptional Immunological and Anticancer Properties of a New Hemocyanin from Fissurella Latimarginata (FLH)

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Mollusk hemocyanins are giant oxygen-transport glycoproteins used in biomedicine because of their potent immunogenic and anticancer properties in mammals, inducing a strong bias to Th1 type immune response. In the search of more powerful and non-toxic adjuvants and immunostimulants in cancer vaccine development, many researchers have utilized hemocyanin properties as carriers of tumor-associated antigens, and by itself, as a non-specific immunostimulant in the therapy of superficial bladder cancer. For more than 40 years Keyhole limpet hemocyanin (KLH) has been used for these purposes, and more recently, we have found in CCH (Concholepas concholepas hemocyanin) similar immunological properties to KLH; however the mechanism underlying hemocyanin antitumor effect is still largely unknown. Here we present the biochemical and immunological properties of a new hemocyanin from the gastropod Fissurella latimarginata, denote as FLH. The characterization of FLH by electron microscopy studies indicated that it shared the same hollow cylindrical form and size of mollusk hemocyanins. By electrophoretic analysis its subunits showed a relative molecular mass near to 350 KDa. In dot blot analysis using biotinylated lectins, FLH demonstrated to have a common glycosylation pattern to KLH that is, a strong reactivity with ConA and PNA, contrary to CCH that showed reactivity only with ConA.

The immunization of different mice strains with these proteins showed that FLH is more immunogenic than KLH and CCH, inducing higher serum antibody titers. In MTT experiments, hemocyanins, per se, demonstrated to reduce significantly the viability of murine (B16F10) and human (MEL1 -2 and -3) melanoma cells, however this effect was significantly more pronounced with FLH. In the B16F10 mouse melanoma model, mice primed with FLH, CCH, KLH and subsequently challenged subcutaneously with B16F10 cells showed that CCH and KLH generated a similar antitumor effect, unlike FLH that was significantly more efficient as demonstrated by the decrease in the tumor growth and prolonged survival. In addition, our experiments confirmed that the pre-immunization with hemocyanins was essential to obtain an antitumor activity.

Thus, this work introduces a new hemocyanin with outstanding antitumor properties with several possible applications in anticancer vaccines development. FONDECYT grant 1050150. S. Arancibia and M. Del Campo are CONICYT doctoral fellows.

IKK Complex and NF-κB: Linkage Between Innate Immune Response and Oncolytic Based Viral Therapy

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The innate host's immune response is believed to negatively affect oncolytic therapy limiting viral replication and to promote tumor rejection during the oncolytic process. Over the past four years, the field of the innate immune response has been highly influenced by the discovery of the IkappaB kinase (IKK)-related kinases, TANK Binding Kinase 1 (TBK1) and IKKi, which regulate the activity of interferon regulatory factor IRF-3/IRF-7 and NF-kappaB (NF-κB) transcription factors. In the present study, we screened several cancer cell lines among the NCI-60 panel for their permissivity to Vaccinia Virus (VACV) and vesicular stomatitis virus (VSV) replication after the treatment with a NBD peptide, which blocks the activation of NF-κB mediated by IKK complex.

Methods: Five hours after treatment with NBD peptide or as negative controls a mutant NBD peptide or DMSO, cells were infected for 18 hours with different strains of VACV or VSV carrying Ruc-GFP. Viral replication was estimated according to GFP expression by FACS analysis by measuring the frequency of

GFP + cells and the geometric mean of fluorescence intensity in the respective population. GFP expression at the protein level was also correlated with the transcriptional activation of GFP mRNA and two VACV transcripts. Finally, cell viability was assessed by Caspase-3 activation assays by FACS analysis.
Results: There was good correlation between GFP expression at protein and mRNA level suggesting that the assay was representative of viral activation. There was also good correlation in the expression of VACV and VSV suggesting that the permissivity of the individual cell lines was not specific to either virus. Surprisingly, blockage of NF-κB activity by the NBD peptide was associated with dramatically decreased viral replication. Analysis on caspase3 activation demonstrated that decreased virally-driven GFP expression was not due to apoptotic phenomena induced by VACV or VSV infection.
Conclusions: VACV and less clearly VSV replication is modulated in cancer cell lines by the NF-κB complex; surprisingly, blockage of NF-κB produced an unexpected reduction in viral replication. Present work is addressing the mechanism leading to this reduction of viral replication by assessing the alterations in the transcriptional pattern of cancer cell lines treated with NBD peptide.

High Functional Competence of Vaccination-Induced Human Self/Tumor-Specific CD8 T Cells

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T cells specific for foreign (eg, viral) antigens can give rise to strong protective immune responses, whereas self/tumor antigen-specific T cells are thought to be less powerful. In deed, most data on ''tumor-specific'' immune responses are actually from T cells specific for non-self antigens. However, the synthetic T cell vaccine composed of antigenic peptides, CpG (PF-3512676) and IFA (Incomplete Freund's Adjuvant) can induce high frequencies of bona fide self-specific circulating CD8 T cells in melanoma patients. Here we analyzed the functionality of these T cells directly ex vivo, by multiparameter flow cytometry. The production of multiple cytokines (IFNγ, TNFα and IL-2) and upregulation of LAMP-1 (CD107a) was efficient and similar to virus-specific T cells. Enhanced CD107a upregulation correlated with strong lytic activity ex vivo. Phosphorylation of STATs was also similar to virus-specific T cells, demonstrating competent activatory signaling pathways. Interestingly, high frequencies of functionally competent T cells were induced irrespective of patient age and gender. These data reveal for the first time that cancer antigen-specific human CD8 T cells can be mobilized by active vaccination to the extent that they become multifunctional in vivo. This is largely different to T cell responses that arise spontaneously or due to vaccination without CpG, which show low T cell frequencies, insufficient cytokine production and cytotoxicity, and deficient signaling. Our findings of robust T cell responses support further development in phase III trials assessing the clinical efficacy of state-of-the-art synthetic T cell vaccines adjuvanted with CpGs.

Investigating the Impact of Autophagy Modulation on Dendritic Cell Cancer Vaccines

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Autophagy has been linked to extended survival when cells are faced with cellular stress. Ex-vivo derived dendritic cells (DCs) undergo substantial stress upon antigen loading and in-vivo delivery and the importance of autophagy in protecting DCs from these stresses is poorly understood. We have employed 2 strategies to investigate the impact of autophagy on DC vaccines. In the first case, we employed rapamycin as an inducer of autophagy in an effort to precondition the DCs prior to infection with recombinant adenovirus (Ad) and recombinant vesicular stomatitis virus (VSV). Kinetic analyses showed that rapamycin was effective at inhibiting its target mTOR within 5 hours of treatment and the inhibition was maintained for at least 24 hours post-transduction. Preconditioning of DCs with rapamycin did not affect infectivity, as measured by GFP expression, nor did it affect maturation, defined by MHC II, CD40, CD86, IL-12 and TNF-α expression. However, we noted a 10-fold reduction in type I interferon secretion following VSV transduction, but not Ad transduction. We are currently conducting in-vivo experiments with rapamycin-preconditioned DC vaccines and results will be discussed at the meeting. In the second case, we have investigated the role of basal autophagy in the context of DC vaccination. We have crossed ATG5fl/fl mice with CD11c-Cre mice to generate conditional knockout mice in which DCs are autophagy-deficient. Results obtained thus far from in-vitro phenotyping experiments of virally transduced DCs indicate that autophagy deficiency does not significantly impact maturation of the cells. The importance of autophagy following delivery of DC vaccines still remains to be determined. This work was supported by grants from CIHR, OCRN and TFF.

The Role of p53G245D Mutant and ZBP-89 in Hepatocellular Carcinoma Cell Death Induced by Histone Deacetylase Inhibitors

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Histone deacetylase inhibitors (HDACi) have emerged as a new class of anti-tumor agents via enhancing the suppressive function of regulatory T cells, and inducing growth arrest and apoptosis. ZBP-89, a zinc finger transcription factor, is involved in HDACi-mediated growth arrest and apoptosis in a number of human cancer cells. ZBP-89 is also known to interact with p53 in hepatocellular carcinoma (HCC). However, the role of p53 mutants in ZBP-89- and HDACi-mediated cell death has not been studied. In this study, we aimed to analyze how efficient was HDACi in induction of cells death in HCC cells with p53 mutants. We first identified and cloned the common p53 mutants in our HCC samples and then focused on the role of one common p53 mutant, p53G245D, in HDACi-mediated cell death/apoptosis in HCC cells. The function of ZBP-89 in p53G245D and HDACi-mediated pathway was examined. Our data first demonstrated that ZBP-89 was essential in HDACi-mediated p21Waf1 up-regulation. We further showed that p53G245D abrogated HDACi-induced p21Waf1 activation, G1 phase arrest and cell death/apoptosis in HCC cells. The inhibitory function of p53G245D was attributed to its ability to directly bind to ZBP-89 and transport it to cytoplasm. By screening relevant cell death pathways, we found that the reduction of HDACi-mediated cell death by p53G245D was associated with the down-regulation of several pro-apoptotic proteins such as caspase 8, Bid and PARP. In conclusion, these data indicate a negative role of p53G245D mutant in regulation of HDACi-mediated HCC cell death/apoptosis via interacting with ZBP-89 and p21Waf1. The finding should provide some guidance in management of HCC patients who have p53G245D mutant. (This work was supported by the Research Grants Council of the Hong Kong SAR. No: CUHK4551/05M and CUHK 462009).

Evaluation of the Efficacy of Nimotuzumab Combined with Radiotherapy in Patients with Advanced Nasopharyngeal Carcinoma

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Objective: To evaluate the efficacy of nimotuzumab combined with radiotherapy in treatment of advanced nasopharyngeal carcinoma.

Methods: Twenty-six untreated patients who have pathologically documented advanced nasopharyngeal carcinoma were randomly divided into combined therapy group and the radiotherapy alone group. All patients in both groups received radical conventionally fractionated radiotherapy to the total dose of DT70-76GY. Nimotuzumab was added for the combined therapy group.
Results: The primary lesion complete remission (CR) rates at the end of treatment for the combined therapy group and radiotherapy alone group were 66.7% and 42.9%, respectively. The relative risk was 1.55 with 95% confidence interval between 1.19 and 2.03. The lymph node complete response (CR) rates at the end of treatment for the combined therapy group and radiotherapy alone group were 80% and 66.7%, respectively. The relative risk was 1.19 with 95% confidence interval (CI) between 1.01 and 1.42. Both data were analyzed by Mantl-Haenszel method and shown significant statistical difference which P value was 0.0007 and 0.03, respectively. The odds ratio of the combined therapy group and radiotherapy alone group for primary lesion and lymph node CR rates were 2.67 (95%CI = 1.50∼4.73) and 2.0 (95%CI = 1.05∼3.8), respectively. Nimotuzumab could increase the 5-year overall survival (OS) rate of the advanced nasopharyngeal carcinoma patients from 50% to 75%. The odds ratio of the combined therapy group and radiotherapy alone group was 3.5 with 95% CI between 1.67 and 7.33. The mean overall survival of the combined therapy group and radiotherapy alone group was 78.42 months and 58.64 months, respectively. The relative risk was 1.34 with 95% CI between 1.13 and 1.59. During the treatment, no patient developed serious adverse events in combined therapy group. No significant differences in radiotherapy-related acute radiation injury between the 2 groups were observed.
Conclusion: Nimotuzumab could improve the CR rate and 5-year OS rate in the combined therapy group. Biological safety of nimotuzumab is well confirmed. Further study should be developed to confirm the clinical value of targeted therapy and nimotuzumab combined with radiotherapy in treatment of advanced nasopharyngeal carcinoma.

Human Melanoma Antigen Specific MHC Class I TCR Engineered CD4 T Cells Undergo Epitope Specific AICD Through an Intrinsic Mitochondria-Centric Process that Involves JNK and p53

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The lack of sufficient tumor antigen specific T cell precursors in most cancer patients, our inability to engage CD4 T cells in cancer immunotherapy protocols, especially in an antigen specific manner, and the potential premature elimination of a significant fraction of tumor reactive T cells through epitope specific activation induced cell death (AICD) represent key limiting factors hindering the development of an effective T cell based cancer immunotherapy. Interestingly, utilizing a melanoma associated antigen specific transgenic TCR isolated from a melanoma patient derived CD8+ cytolytic T lymphocyte (CTL) line, we have recently shown that with TCR engineering approach, we can not only generate sufficiently large numbers of customized tumor reactive CD8+ CTL, but also can program human CD4 T cells to function as MHC class I directed simultaneous "helper as well as lytic effectors" (Chhabra et al, JI, 2008, Ray S. Chhabra et al, *J Clin Immunol*. 2010). We here show that these MHC class I TCR driven CD4 T cells are also susceptible to undergo epitope specific AICD, just like CD8+ CTL.
We also show that the AICD in these TCR engineered CD4+ T cells is a death receptor-independent, caspase-independent, intrinsic process that involves the activation of JNK and blocking JNK could rescue a substantial fraction of these CTL from undergoing AICD, similar to our findings with the natural CD8+ CTL (Chhabra et al. *EJI*. 2006 and Mehrotra et al. *JI*. 2004). Furthermore, we also show that p53 plays a critical role in the MHC class I TCR driven, mitochondria-centric AICD of TCR engineered CD4 T cells. These findings have implications for T cell based cancer immunotherapy protocols.

Generation and Functional Characterization of Breast Cancer Antigenic Epitope Specific Anti-Tumor T Cell Response

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Engaging T cell immunity is critical for developing an effective immune based cancer therapy. However, development of an effective anti-tumor T cell response faces several inherent constraints. Among these includes, the lack of sufficient tumor antigen specific T cell precursors in most cancer patients, immune regulatory/ suppression mechanisms, and the potential premature elimination of a significant fraction of tumor reactive T cells through epitope specific activation induced cell death (AICD). We have recently developed several methodologies to generate robust and long lasting natural anti-tumor T cell responses by expanding the host inherent natural anti-tumor T cell precursors, in human melanoma model (Chhabra et al. *J Clin Immunol*. 2008; Chhabra et al. *EJI*. 2004). We have also developed a TCR engineering based approach to create customized MHC class I TCR driven anti-tumor CD4 and CD8 T cells (Chhabra et al. *JI*. 2008, Ray et al. *J Clin Immunol*. 2010). Furthermore, we have also shown that the activation induced cell death (AICD) in CD8+ human anti-tumor cytolytic T lymphocytes (CTL) is a caspase-independent, DR-independent, JNK driven, intrinsic process, and blocking JNK interferes with this AICD process (Chhabra et al. *EJI*. 2006; Mehrotra et al. *JI*. 2004).
We here present our work on generation and functional characterization of breast cancer antigenic epitope specific T cell response. Our data show that we can effectively generate Her-2/neu Her-2/neu369-377, and mucinase-1 (MUC-112-20) epitope specific CTL response, and these CTL exhibit antigen specific effector function. This will now allow us to develop strategies to create long lasting, AICD resistant breast cancer specific CTL, and also to isolate breast cancer epitope specific TCR genes to create customized breast cancer reactive anti-tumor T cells.

Immunobiologic Activity of the Demethylating Dinucleotide SGI-110

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Epigenetic alterations play a major role in human malignancies by affecting crucial cellular pathways in cancer initiation and progression (eg, cell cycle control, apoptosis, invasive and metastatic potential, angiogenesis). In this context, we have demonstrated a key role of aberrant DNA hypermethylation in favoring tumor escape from host's immune recognition, through the down-regulation of different components of the "tumor recognition complex" (ie, HLA class I antigens, tumor-associated antigens belonging to the cancer/testis antigens (CTA) class and accessory/co-stimulatory molecules) in neoplastic cells of different histotypes. These findings contribute to explain at least in part the reduced clinical efficacy of immunotherapeutic approaches for cancer treatment. The present study was designed to evaluate the immunomodulatory activity of the new DNA hypomethylating agent SGI-110, a dinucleotide of 5-aza-2′-deoxycytidine and guanosine, in different solid malignancies. Five cutaneous melanoma, 2 mesothelioma, 2 renal cell carcinoma and 2 sarcoma cell lines were treated in vitro with 1 μM SGI-110, added every 12 hours for 2 days (4 pulses), or treated for 6 days with addition of new drug at day 3. RT-PCR analyses showed that treatment with SGI-110 induced/up-regulated the expression of a large panel of CTA analyzed (ie, MAGE-A1, -A2, -A3, -A4, -A10, GAGE 1-2, GAGE 1-6, NY-ESO-1, SSX 1-5) in all investigated cell lines. Consistently, quantitative real-time RT-PCR analyses of the CTA MAGE-A3 and NY-ESO-1, which are currently utilized as therapeutic targets in clinical trials of CTA-based cancer vaccination, demonstrated

that SGI-110 strongly up-regulated their constitutive levels of expression in neoplastic cells of all investigated histotypes. This latter observation was confirmed at protein level by flow cytometric analysis of the intracytoplasmic levels of CTA, in melanoma cells. Concomitantly, flow cytometric analyses revealed that treatment with SGI-110 up-regulated the expression of HLA class I antigens, HLA-A2 allospecificity and the co-stimulatory molecule ICAM-1. Altogether, these preliminary in vitro experimental evidences strongly suggest that SGI-110 may represent an attractive therapeutic agent to comprehensively increase immunogenicity and immune recognition of neoplastic cells from solid tumors, and provide the scientific rationale for its clinical development to design new and more effective combined chemo-immunotherapeutic approaches in patients with solid malignancies.

4-1BB Activation Induces the Master-regulator Eomes and A Broad-spectrum TH1 Phenotype Which Synergizes with CTLA-4 Blockade to Reject B16 Melanoma

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Antibodies which block the co-inhibitory receptor CTLA-4 or which activate the co-stimulatory receptor 4-1BB can promote the rejection of some murine tumors, but fail to cure poorly immunogenic tumors like B16 melanoma. We find that combining these two antibodies in the context of a Flt3-ligand, but not a GM-CSF, based B16 melanoma vaccine promoted synergistic levels of tumor rejection. 4-1BB activation elicited strong infiltration of CD8+ T cells into the tumor and drove the proliferation of these cells, while CTLA-4 blockade did the same for CD4+ effector T cells. Anti-4-1BB depressed regulatory T cell infiltration of tumors and this effect was dominant over the tendency of αCTLA-4 to expand them. 4-1BB activation strongly stimulated TH1-type inflammatory cytokine production in the vaccine and tumor draining lymph nodes as well as in the tumor itself. The addition of CTLA-4 blockade further increased IFN-γ production from CD4+ effector T cells in the vaccine draining node and the tumor.

A hallmark of 4-1BB agonist antibody treatment is the upregulation of the lectin KLRG1 on tumor infiltrating CD8+ and CD4+ T cells. We find that these KLRG1+ T cells in the tumor express high levels of multiple killing-associated genes and may be responsible for the increased anti-tumor cytotoxicity which has been attributed to α4-1BB treatment. Further investigation revealed that formation of these cells is driven by high-level expression of the master-regulatory transcription factor Eomesodermin (Eomes) in both the CD8+ and CD4+ T cell compartments. To determine the pathway leading from 4-1BB agonist antibody to induction of Eomes expression, we have characterized the direct effects of α4-1BB on cytokine production from antigen presenting cells. Further, we have used a series of gene-specific knockout mice to validate candidate cytokines and transcription factors involved in this pathway. These findings reveal a previously unappreciated role for Eomes in generating extremely potent tumor-specific effector T cells which may be critically important for understanding and augmenting the effects of TNF-receptor family agonist antibodies as well as for T cell adoptive transfer therapies.

Clinical Efficacy of the Anti-Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Monoclonal Antibody Ipilimumab in Pretreated Metastatic Uveal Melanoma Patients

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Background: The fully human anti-CTLA-4 monoclonal antibody ipilimumab potentiates anti-tumor T cell responses. Ipilimumab has improved overall survival (OS) of advanced cutaneous melanoma patients (pts) in a phase III trial; however, no data are available of its clinical effectiveness in uveal melanoma where no effective treatment is available. We report the European experience (6 Institutions) with ipilimumab in metastatic uveal melanoma pts.

Methods: Thirteen stage IV pts (8 male, 5 female), median age 57 (30 to 76) years, ECOG performance status 0 to 1, with uveal melanoma progressing to 2 median (1 to 4) previous therapies for metastatic disease received ipilimumab within an Expanded Access Program. All pts had history (1) or evidence (12) of liver metastases, 1 of brain metastases and 3 of elevated (> 1 × upper limit of normal) LDH. In the induction phase (IF) pts received ipilimumab (10 mg/Kg i.v.) q3 weeks (wk × 4 cycles; after a 12 weeks rest, treatment was repeated q12 weeks in the maintainance phase (MF) starting from week (wk) 24. Tumor assessment (TA) per modified WHO criteria was evaluated at baseline, at weeks 12 and 24, then q12 weeks. Adverse Events (AE) and immune related AE (irAE) were collected according to Common Terminology Criteria for Adverse events version 3.0.

Results: All pts received at least one ipilimumab dose, 9/13 completed all IF cycles while the remaining 4 pts were prematurely withdrawn for disease progression; 5 pts entered the MF. No objective tumor responses were observed; however, TA at wks 12, 24 and 36 showed stable disease (SD) in 2/9, 3/6 and 1/4 pts, respectively. As reported for metastatic cutaneous melanoma pts, slow, steady tumor volume decline and appearance of new lesions that subsequently shrunk was observed. No grade 3/4 AEs were reported. Three pts (23%) had grade 3 IrAEs (1 trombocytopenia, 1 diarrhea, 1 ALT/AST elevation) that resolved after steroid therapy. Median OS as of March 1, 2010 was 36 weeks (range 2 to 102 wk). One patient, maintaining SD at > 2 years from initial ipilimumab administration, is still on treatment.

Conclusions: Ipilimumab treatment of metastatic uveal melanoma pts is feasible and safe. A sizeable proportion of pts experienced prolonged SD and extended survival. These evidences, strongly identify uveal melanoma as a promising indication for ipilimumab treatment to be investigated in phase II trials.

Monoclonal IgE Targeting Prostate Specific Antigen (PSA) or HER2/NEU (HER2) as a Tumor Specific Immunotherapeutic Strategy

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Biological treatments of both breast cancer (BCa) and prostate cancer (PCa) have proven efficacy, however these malignancies continue to be leading causes of cancer deaths worldwide. To improve the treatment of BCa and PCa, we have developed two monoclonal IgE antibodies specific for HER2 and PSA respectively. HER2 is cell surface antigen over-expressed in 30 percent of BCa and PSA is a secreted antigen that accumulates locally in prostate tissue and PCa. Both antigens have been used for tumor targeting and both are associated with circulating forms (secreted PSA & shed extracellular domain of HER2 (ECDHER2)). We hypothesized that an IgE mediated local acute inflammatory response in the tumor microenvironment would result in tumor destruction and that a subsequent adaptive anti-tumor immune response that would lead to further elimination of disease. We also hypothesized that IgE through interaction with Fcε bearing antigen presenting cells could facilitate antigen processing and cross presentation of PSA or HER2 to enhance the adaptive cellular immune response. To target HER2 we used the variable regions of

the scFv C6MH3-B1 obtained from a human phage library and to target PSA we used the variable regions of a murine anti-human PSA antibody AR47.47. In both cases, the DNA encoding the variable regions were cloned into either the human κ light chain or human ε heavy chain expression vectors to yield the fully human anti-HER2 IgE and the mouse/human chimeric anti-PSA IgE. Both IgE antibodies, expressed in murine myeloma cell lines, are properly assembled and secreted, bind antigen (determined by ELISA, immunoprecipitation, or flow cytometry), and bind cell-surface FcεR (detected by flow cytometry). Anti-HER2 IgE significantly blocked the proliferation of SK-BR-3 human BCa cells in vitro. In addition, antigen presentation assays using human dendritic cells loaded with anti-HER2 IgE complexed with ECDHER2 and with anti-PSA IgE complexed with PSA showed enhancement in the percentage of interferon-γ producing CD4 and CD8 T cells as compared to priming with antigen alone. Both antibodies have initial evidence of anti-tumor activity. A pilot intravenous infusion of anti-HER2 IgE in Cynomolgus monkeys who have FcεR that interact with human Fcε was well tolerated. Consistent with expected tissue redistribution, the infused antibody was absent from the circulation within one week. Both antibodies are capable of enlisting a potent range of human effector cells and show promise for the immunotherapy of solid malignancies.

The Role of Interleukin-12 in Modulating the Production of Interleukin-18 and Interferon-Gamma in Patients with Breast Cancer: Correlation to Clinicopathological Data

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Breast cancer is an important public health problem. It is the commonest form of cancer in women throughout the world. Previous reports have indicated that out-come of malignant neoplasia in humans is often accompanied by defective cellular immunity. To induce more effective antitumor immune response, increasing the producton of Th1-cytokines are needed. The aim of this work was to investigate the regulatory effects of recombinant interleukin-12 (rIL-12) on interleukin-18 (IL-18) and interferon-gamma (IFN-γ) production in patients with breast cancer as correlated with clinicopathological data.

Peripheral blood mononuclear cells were isolated from all patients and cultured without and with rIL-12 supplementation. The levels of IL-18 and IFN-γ in culture supernatants were detected before and after IL-12 addition using enzyme- linked immunosorbant assay. Statistical analysis of data revealed that, the supplementation of cultures with IL-12 significantly increases the mean values of both IL-18 and IFN-γ in different clinicopathological parameters. In univariate analysis by age, tumor size, grade, number of lymph nodes, ER-BR, HER2 neu and IL-18 level, It was found that patients with IL-18 level higher than median at the time of diagnosis had higher survival results than those with lower value. In multivariate analysis the tumor size and HER2 neu were found to have independent prognostic value ($P < 0.001$) but IL-18 did not have an independent influence on the survival concolusion. From our study we can conclude that, Interleukin-12 plays a crucial role in the improvement of impaired immunity by enhancing the production of IL-18 and IFN-γ in patients with breast cancer. This could result in new therapeutic approaches for correction of the immunodeficiency associated with malignancy.

Th17 Cells in Melanoma and Renal Cell Cancer Patients

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Despite the well-studied role of Th17 cells in the pathogenesis of several pro-inflammatory and autoimmune diseases, their function in cancer immunity remains controversial. We have previously reported increased frequencies of these cells in hormone-resistant prostate cancer patients compared to healthy controls and demonstrated that patients with longer time to disease progression possessed lower levels of Th17 cells pre-vaccination. Here, we have studied the frequency and cytokine profile of Th17 cells in renal cell cancer (RCC) and melanoma patients and correlated pre-treatment frequency of these cells with clinical outcome after vaccination using peptide (RCC, in a phase II study investigating the multi-peptide cancer vaccine IMA901) or mRNA encoding four tumor antigens (melanoma). Compared to healthy controls, there were increased frequencies of IL-17-producing T cells in the blood of patients with either RCC or melanoma prior to vaccination ($P < 0.001$ and $P < 0.01$, respectively). There was also a significant accumulation of CD4+ T cells producing IL-10 in both ($P < 0.001$ and $P < 0.05$, respectively), whereas no such difference could be observed for IFNγ- and TNF-producing CD4+ T cells. Next we determined the cytokine repertoire of Th17 cells in these patients compared to healthy controls using 10 color flow cytometry. The majority of Th17 cells also produced TNF in both patients and controls. However, in cancer patients, a higher proportion of Th17 cells were double-positive for IFNγ and TNF ($P < 0.05$). Despite increased levels of Th17 cells in RCC and melanoma patients, no significant correlation between pre-treatment frequency of these cells and clinical outcome after vaccination was observed. Thus, the negative impact of Th17 cells on prognosis that we previously observed in prostate cancer patients might be specific to the cancer type or the immunotherapy setting and not be a general phenomenon.

Early Development of CDX-1401, a Novel Vaccine Targeting NY-ESO-1 to the Dendritic Cell Receptor DEC-205, in Combination with Toll-Like Receptor (TLR) Agonists

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Background: Antibody targeting of antigens to the DEC-205 molecule on dendritic cells can elicit potent immunity in preclinical models. We generated a new human antibody, 3G9, with high affinity and specificity for human DEC-205. The NY-ESO-1 cancer-testes antigen was genetically attached to the C-terminus of the 3G9 heavy chain and the fusion protein (CDX-1401) was manufactured using CHO cells. Unlike NY-ESO-1 protein, DEC-205 targeted NY-ESO-1 is efficiently cross-presented to CD8 T cells in vitro and efficiently elicits both CD4+ and CD8+ NY-ESO-1-specific T cells against multiple epitopes from lymphocytes of cancer patients.

Methods: A Phase 1/2 dose-escalation study to assess the safety, immune response, and anticancer activity of CDX-1401 in patients with advanced malignancies that have progressed after any available curative/salvage therapies. CDX-1401 is administered intracutaneously in combination with the TLR7/8 agonist, resiquimod (250 mg of 0.2% gel, topically, daily × 2), once every two weeks × 4. Additional treatment cycles are permitted until intolerance or progression.

Results: 19 patients have been enrolled: melanoma (n = 14), ovarian (n = 2), myeloma (n = 1), NSCLC (n = 1) and leiomyosarcoma (n = 1); median age = 62; male = 63%. CDX-1401 dose levels received: 0.1 mg (n = 7), 1 mg (n = 6) and 3 mg (n = 6). 5/16 (31%) of patients with tumor samples assessed by IHC demonstrated tumoral NY-ESO-1 expression. There have been no severe treatment-related toxicities; mild to moderate injection site reactions (rash, pruritus, pain, erythema) and fatigue are most

common. CD4+ and CD8+ NY-ESO-1-specific T cell responses and high titer anti-NY-ESO-1 IgG responses (some > 1:200,000) develop frequently following treatment with CDX-1401. Enhancement of pre-existing anti-NY-ESO-1 titers in patients with tumoral expression of NY-ESO-1 has been observed. 6 patients with stable disease (range: 5.3+ to 8.5+ mo) have been retreated, including 3 patients that went on to a third cycle. Of the patients with stable disease, the majority developed NY-ESO-1-specific immune responses and 2/5 with tested tumor samples were found to express NY-ESO-1.

Conclusions: CDX-1401 is well tolerated and induces robust NY-ESO-1 immunity in advanced cancer patients. Additional cohorts examining combination treatment with the MDA-5/TLR3 agonist, poly-ICLC, are planned.

Unraveling the Paradoxes of ATM Resensitized Dynamics in LNCaP Cell Line via Epigallocatechin-3-Gallate (EGCG)

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Epigallocatechin-3-gallate (EGCG) is a major ingredient of green tea (GT) presumably holds a potential to prevent pathogenomics. Prostate cancer aggressiveness is triggered by fusion transcripts formed because of genomic instability induced by juxtapositioning of two genes. An abolished Ataxia Telangiectasia mutated kinase (ATM) dynamics is incapable of safeguarding integrity of DNA. In agreement with this assumption ATM and DNA dependant Protein Kinase (DNA-PK) were impaired in LNCaP cell line to confirm a tight interaction of ATM and DNA-PK with the expression profile of Transmembrane Protease Serine 2 and ETS related gene (TMPRSS2-ERG). Abolished ATM proved instrumental to expression of the fusion transcript. Similarly blunting of DNA-PK down regulated the expression of the fusion transcript giving a notion that it is involved in the chromosomal translocation. LNCaP cell lines were analyzed for the effect of EGCG on the expression profile of TMPRSS2-ERG. In this particular unprecedented study treatment of the LNCaP cell line with EGCG recapitulated ATM expression and activity and downregulated the fusion transcript generation. These results underscore the therapeutic effect of EGCG in attenuating the exacerbation of the disease.

Immune Response Against DKK1 in Common Cancers

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Cancer immunotherapy has demonstrated promising results for advanced melanoma. Furthermore, adoptive transfer of tumor-specific T lymphocytes combined with new cytokines and peptide immunization from well defined tumor antigens (TA) may lead to even greater success in the next generation of clinical trials. Currently, such approaches are, however, difficultly transposable to common malignancies such as lung and breast cancers due to differences in the immunological response generated against these tumors. Furthermore, suppression mechanisms must be better defined and controlled, expansion of tumor-specific T lymphocytes must be optimized and new TA from common cancers must be identified. We have previously reported Dickkopt-1 (DKK1) mRNA overexpression in 50% of lung cancer specimens and in 29% of breast cancer samples, half of them being estrogen and progesterone receptor-negative (Forget et al. *Br J Can*. 2007). We further confirmed DKK1 expression in the placenta with weak or negative expression detected in an extensive panel of normal tissues. This expression profile allowed us to hypothesize that DKK1 could constitute a good TA candidate for common cancer immunotherapy.

To confirm our hypothesis, we carried out in vitro stimulations of peripheral blood mononuclear cells (PBMC) from lung and breast cancer patients. DKK1 synthetic peptides predicted for their capacity to be presented (Parker & SYFPEITHI Algorithms) by major histocompatibility complex (MHC)-A*0201 were synthesized and MHC class I stabilization assays were performed to confirm peptide binding capacity. Multiple rounds of stimulation were completed using DKK1 synthetic peptides and specificity of subsequently expanded T lymphocytes was confirmed by enzyme-linked immunosorbent spot (ELISPOT). This method has enabled us to identify, isolate and characterize DKK1-specific T lymphocytes. Cytokine secretion profile of anti-DKK1 T lymphocytes was established by cytokine mutiplex assay (Bio-Rad). Production of cytokines such as IP-10 (CXCL10), MIP-1β and GM-CSF confirmed expansion of polyfunctional DKK1-specific T lymphocytes. Based on these results, we believe that anti-DKK1 T lymphocytes could be exploited in common cancer immunotherapy.

Development of a New Formulation to Enable Sustained IL-2 Release In Vivo

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Introduction: High-dose Interleukin-2 (IL-2) is an FDA approved therapy for the treatment of malignant melanoma and renal cancers. IL-2 is administered systemically (i.v.) and leads to disease regression in 15% to 20% of stage IV metastatic melanoma patients. However, IL-2 is associated with grade 3 and 4 side effects and this significantly impacts its therapeutic use. The development of alternative administration strategies is limited by IL-2 instability in solution. In recent years a family of low-melting point salts, termed "ionic liquids", have gained attention for their ability to increase protein stabilization in solution.

Aims: The aim of the current study was to determine if choline dihydrogen phosphate (C-DHP) could function as a stabilizing excipient for IL-2 in liquid formulation.

Methods: The effect of C-DHP on IL-2 thermal stability was measured between 15°C and 75°C by circular dichroism at 222 nm. The cytotoxicity of increasing concentrations of C-DHP was measured in mouse splenocytes and B16-F10 melanoma cells by trypan blue exclusion and resazurin reduction assay. The biological activity of IL-2 and C-DHP-IL2 was determined by flow cytometry using HT-2 T cells, a cell line that requires IL-2 to survive and proliferate.

Results: C-DHP at final culture medium concentrations of 1 to 30 mM was not cytotoxic to splenocytes or B16-F10 melanoma cells. However at C-DHP concentrations of > 35 mM, C-DHP caused significant culture medium acidification and cell cytotoxicity, effects that were abrogated by the addition of sodium bicarbonate. Under pH-buffered conditions (final culture medium pH7.2-4), 30 mM C-DHP did not significantly modify the IL-2 melting (unfolding) temperature (without C-DHP Tm = 53.9°C; with CDHP Tm = 52.3°C) or IL-2-dependant HT-2 cell proliferation. However, when HT-2 cells were stimulated to undergo proliferation (fetal bovine serum; 5% or 10% v/v), 30 mM C-DHP-IL-2 significantly improved HT-2 cell viability as compared to cells treated with IL-2 alone.

Conclusions: IL-2 is readily soluble in a C-DHP ionic liquid. pH buffering is required when (C-DHP) > 30 mM and, when used in a buffered solution, C-DHP significantly improves IL-2 activity in vitro. Further studies will determine whether this approach can be used to improve IL-2 activity, and decrease systemic side effects, in vivo.

Histone Modification Upregulates Expression of IL-13Rα2 in Human Pancreatic Cancer Cells and Enhances the Effectiveness of IL-13 Receptor Targeted Immunotoxin IL-13-PE in Animal Model of Human Pancreatic Cancer

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Interleukin 13 Receptor α2 (IL-13Rα2) is a cancer-associated antigen and a target for receptor directed anti-cancer therapy. We have reported that a recombinant immunotoxin, consisting of IL-13 and truncated Pseudomonas exotoxin (IL-13-PE) is highly cytotoxic to tumors that express high density IL-13Rα2 in vitro and in vivo. However, IL-13-PE is not very effective in tumors that express low level of IL-13Rα2. Herein, we have examined whether IL-13Rα2 can be modulated by epigenetic histone modification in human pancreatic cancer cells. When eleven pancreatic cancer and three normal (two epithelial and one fibroblast) cell lines were treated by three different histone deacetylase (HDAC) inhibitors (trichostatin A, sodium butyrate and Suberoylanilide hydroxamic acid), we found that HDAC inhibitors significantly increased mRNA and protein levels of IL-13Rα2 in low-IL-13Rα2 expressing pancreatic cancer cells, compared to no or marginal upregulation in high-IL-13Rα2 expressing pancreatic cancer and normal cells. HDAC inhibitors enhanced anti-cancer effect of IL-13-PE in vitro in low-IL-13Rα2 expressing pancreatic cancer cell lines by lowering IC50 (concentration of IL-13-PE that kills 50% of the cells in protein synthesis inhibition assay) from >1000 ng/mL to 40 to 50 ng/mL. Similar effects did not occur in high-IL-13Rα2 expressing tumors or in normal human cell lines. In xenograft mouse models developed by implanting low-IL-13Rα2 expressing cell lines (Panc-1 and ASPC-1), IL-13-PE alone showed no anti-cancer effect, but in combination with HDAC inhibitors (trichostatin A and Suberoylanilide hydroxamic acid), IL-13-PE significantly decreased tumor burden and prolonged survival of animals. Trichostatin A increased IL-13Rα2 expression in tumors approximately 500 times compared to vehicle treated mice with Panc-1 tumors. A combination therapy with HDAC inhibitors and IL-13-PE had marginal or no detectable histological changes in any vital organs of mice, which indicate that this effect is tumor specific restricted within tumor compartment. Taken together, we have identified a new approach to target pancreatic cancer by combining HDAC inhibitors with a potent immunotoxin IL-13-PE. Similar approach may be useful for other malignancies as well.

COX2 Blockade Suppresses Gliomagenesis by Inhibiting CCL2-Mediated Accumulation of Myeloid-derived Suppressor Cells in Glioma Sites

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A line of epidemiologcal studies has suggested that the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with reduced glioma occurrence in humans. NSAIDs mediate their biological effects at least partially by suppressing cyclooxygenase (COX) 2 and its product prostaglandin (PG) E_2, which induces immunoregulatory cells including myeloid-derived suppressor cells (MDSCs). PGE_2 also impacts chemokines, such as down-regulation of CXCL10 production by dendritic cells. Based on these findings, we hypothesized that COX2 blockade by NSAIDs would suppress gliomagenesis by inhibiting MDSC accumulation in gliomas. We chose acetylsalicylic acid (ASA) for immunological evaluation because it did not inhibit the growth of cultured glioma cells in vitro at pharmacological concentrations in contrast to selective COX2 inhibitors. To address the impact of COX2 blockade on gliomagenesis, we induced de novo gliomas in mice by trasposon-mediated intracerebroventricular transfection of oncogenes. Daily ASA treatment inhibited systemic PGE_2 production in wild type (WT) mice with developing gliomas. The treatment prolonged the survival of these mice only when started simultaneously with glioma induction. Glioma tissues of the ASA-treated mice exhibited a decrease in mRNA for the MDSC-attracting chemokine Ccl2 but an increase of the CTL-attracting chemokine Cxcl10. Consistently, analyses of brain tumor-infiltrating leukocytes in the ASA-treated mice revealed a decrease of $CD11b^+Ly6G^+$ cells but an increase of $CD8^+CD107a^+$ cells compared with those in non-treated mice. Cox2-deficient mice with developing gliomas corroborated these phenomena. Ccl2-deficient mice with developing gliomas exhibited a prolongation of survival with a decrease in intratumoral MDSC accumulation. In addition, these mice exhibited an increase in Cxcl10 and accumulation of CD8+ cells in glioma sites. Antibody-mediated MDSC depletion for WT mice with developing gliomas exhibited an increase in Cxcl10 and prolonged survival, suggesting an impact of MDSCs on glioma development. Cxcl10-deficient mice exhibited shortened survival with a decrease in CD8+ cell accumulation, suggesting the significance of CXCL10 in intratumoral T cell accumulation. Collectively, these findings indicate important roles of COX2 pathway in gliomagenesis through CCL2-mediated MDSC accumulation and reduction of CXCL10-mediated CTL accumulation in glioma sites. These data also support development of NSAID-including strategies for glioma immunotherapy.

Elevated MDSCs in Pancreatic and Esophago-gastric Cancer Patients Correlates with Poor Prognosis

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Background: Myeloid derived suppressor cell (MDSC) and regulatory T cell (Treg) accumulation in cancer patients is proposed as an important mechanism of tumor immune evasion. We present for the first-time analysis of both MDSCs and Tregs in pancreatic and esophago-gastric cancer.

Methods: Peripheral blood samples were collected from 35 pancreatic, 46 esophageal and 19 gastric cancer patients and 31 normal healthy volunteers. PBMC was harvested and stored in liquid N2 with subsequent flow cytometric analysis of MDSC (HLADR- Lin1- $CD33^+$ $CD11b^+$) and Treg (CD4+ CD25+ CD127low/- FoxP3+). Unpaired *t* tests were applied to analyse MDSC and Treg levels between groups. Kaplan-Meier survival analysis was performed to compare patients with normal MDSC levels (as defined by the controls range, 0% to 2% total PBMCs) and high MDSCs (> 2%).

Results: The mean percentage of MDSCs in each cancer group was significantly higher than controls; pancreatic 2.455% ($P = 0.0005$), esophageal 1.623% ($P = 0.0260$) and gastric 2.029% ($P = 0.0326$), with controls 1.104%. Pancreatic and esophago-gastric cancer patients had significantly higher mean Treg percentages than controls, $P = 0.0036$ and $P = 0.0189$ respectively. There was a significant positive correlation between upper GI cancer patients' MDSC and Treg scores (Spearman's rho +0.285; $P = 0.008$). Patients with normal MDSC levels survived longer than those with high levels (log rank: $P = 0.011$), median survival 231 versus 145 days. The influence of MDSC score was further quantified by Cox regression analysis with MDSC score as a covariate: $P < 0.0005$. Treg percentage was not significantly associated with survival. Conclusions: Pancreatic and esophago-gastric cancer patients have elevated MDSC correlating with poor prognosis, indicating MDSCs as a potential biomarker for survival.

Summary Analysis of Brain Cancer Patients Treated with Pritumumab

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Pritumumab is a human IgG1 kappa antibody that has been derived from a B-cell isolated from a regional draining lymph node of a patient with cervical carcinoma. Specificity analysis of the

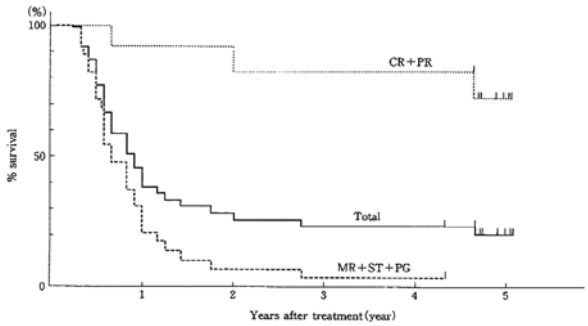

FIGURE 1. Five-year survival data of 66 glioblastoma patients treated with pritumumab. CR indicates complete response; MR, moderate response; PG, progressive disease; PR, partial response; ST, stable disease.

antibody with human tissues showed the antigen, altered tumor-associated vimentin, to be highly restricted to various cancers and not normal cells and tissues. In various clinical trials in Japan 249 patients with brain cancer were treated with pritumumab. The overall response rate was between 25-30% with several survivors beyond 5-years post-treatment. In one Phase II study of 66 patients the survival rate of complete and partial responders was over 70%. The patients were on a low dose regimen of 1 mg given twice a week for a course of 24 weeks for a total dose of 48 mgs per course. Pritumumab appears to be a safe and effective therapy in patients with malignant gliomas (Fig. 1).

Gene Expression Signature Allows Prediction of NK cell Content in Tissue and Survival of Renal Cell Carcinoma Patients

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Renal cell carcinoma (RCC) is considered immunogenic based on the observation of spontaneous regression and response to immunotherapy. However, only subgroups of patients are responders and the reason why these patients respond whereas others exhibit tumor progression under identical cytokine therapies is largely unknown. RCC tissues are densely infiltrated with immune effector cells but how the infiltrate relates to tumor growth-control or patient's responsiveness to therapy is unknown. We previously showed that RCC tumors can be separated into two groups according to their percentage of NK cells within tumor-infiltrating lymphocytes (TIL): one group has a high (NKhigh), the other a low (NKlow) percentage of NK cells (cut-off: 20% NK within TIL). Additionally, the functional quality of NK cells differed, with NK cells from NKhigh tumors gaining cytolytic activity by short-term incubation with low-dose IL-2. Based on this quick responsiveness to IL-2, we considered that the amount of NK cells among TIL may be a predictive marker for clinical outcome and could correlate with survival and response to immunotherapy. Testing this hypothesis requires the analysis of a large patient cohort, including patients with relevant follow-up data after IL-2 immunotherapy. The current procedure for NKhigh/NKlow discrimination utilizes flow cytometry of TIL isolated from fresh post-surgery tissue and, thus, is not compatible with the analysis of archived tissues. Therefore, we sought to identify a molecular tissue-profile that reproduces the flow cytometry-based quantification of NK-content of TIL. Using qRT-PCR, transcript levels of lymphocyte markers, chemokines and cytokines were quantified in RCC tissues with predetermined NK cell-content of TIL. Transcript levels of NKp46, perforin, CX3CL1 and its receptor CX3CR1 were found to correlate significantly with the flow cytometry-based classification. Patients with tumors showing high CX3CR1 transcript level had significantly better survival than patients with low CX3CR1 transcript levels. The new gene profile, being easily amenable to clinical translation, offers the possibility to investigate the relevance of NK cells in the prediction of cancer progression or the response to immunotherapy.

Enhanced Anti-tumor Effect of Combination Therapy With Anti-CD40 Antibody and the mTOR Kinase Inhibitor AZD8055

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mTOR signaling, which plays a central role in controlling cell proliferation, survival, mobility and angiogenesis, is considered an important target for new anticancer drugs development. The involvement of the CD40/CD154 signaling pathway has also been reported to play an important role in regulating anti-tumor immune responses. In this study, using a first-in-class orally bioavailable mTOR kinase inhibitor, AZD8055, which, unlike rapamycin, can inhibit both mTORC1 and mTORC2, we evaluated the anti-tumor efficacy of mTOR kinase inhibitor treatment alone in a murine liver metastatic renal cell carcinoma (Renca) model, and compared biological responses achieved by administering AZD8055 as a single agent to those obtained in combination with agonistic anti-CD40 antibody. In vitro survival assays showed that Renca cell apoptosis could be induced by AZD8055. In vivo, although AZD8055 alone modestly inhibited Renca tumor development in the liver, its use in combination with anti-CD40 induced significantly greater anti-tumor responses. The combination treatment also resulted in an increase and activation of macrophages, dendritic cells, NK cells and CD8 T cells in liver. AZD8055/anti-CD40 treatment also increased the level of systemic Th1 cytokines, including IL-12, IFN-γ, TNFα. IFN-γ production by CD8+ T cells and TNFα production by macrophages were also found to be elevated in the AZD8055/anti-CD40 combination treated-group, compared with any single treatment group. Using IFN-γ KO mice, we demonstrated that the AZD8055/anti-CD40- induced anti-tumor response was dependent on IFN-γ. Furthermore, the expression of Th1-associated chemokines, RANTES, MIG and IP-10, were significantly induced in the combination treated-group. These data indicate that the combination of mTOR kinase targeting agents with immunotherapy results in synergistic immune responses and suggest that this combination approach could be an area of further development in renal cancer therapy.

Developing a Neoadjuvant Murine Surgical Model of Cancer Using Vesicular Stomatitis Virus

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Introduction: Surgery is the primary treatment modality for most solid tumours. Despite complete resection, the development of metastatic disease limits its curative potential and provides the rationale for neoadjuvant (preoperative) therapies. Oncolytic Viruses (OVs) are replicating therapeutics that are selected or engineered to grow in malignant cell types and are capable of killing the infected target, while leaving normal, adjacent cells unharmed. OV infection creates localized inflammation and

additionally the presentation of foreign viral proteins together with tumour antigens, promoting adaptive anti-tumour immune responses. We have shown induction of an immune response by using Vesicular Stomatitis Virus (VSV) as an adjuvant in a tumour lysate vaccine setting. We hypothesize that the treatment of tumours prior to surgical resection will generate an in situ anti-tumour immune response which may protect immune competent hosts from recurrence or metastasis.
Methods: Donor mice are injected with $1 \times 10\text{^}6$ CT26lacZ tumour cells subcutaneously into both flanks on day -14. Mice are euthanized on day 0 at which point the tumours are removed. Recipient mice receive 1×1 mm CT26lacZ tumour implants subcutaneous in the flank. Mice are treated with PBS or $5 \times 10\text{^}8$pfu VSV51ÄGMCSF intravenous prior to complete surgical resection of the flank tumour. Mice are then challenged with $1 \times 10\text{^}6$ CT26lacZ cells subcutaneous on the opposite flank, and tumour growth and rate is recorded.
Results: We developed a CT26lacZ surgical model of cancer, where mice that received a 1×1 mm tumour implant from donor mice grew the challenge tumour more consistently compared to mice that received a subcuteneous injection of CT26lacZ cells (no treatment). We found that mice treated with PBS or VSVΔ51GMCSF prior to surgical resection were not protected against their challenge tumour. Mice that were treated with VSVΔ51GMCSF without surgical removal of the primary tumour were protected against the challenge tumour. On the contrary, mice that were treated with VSVΔ51GMCSF and received a mock surgery or complete surgical resection were not protected against their challenge tumour.
Conclusions and Discussion: Our data demonstrates that surgical stress abrogates protection against the challenge tumour and therefore the anti-tumour immune response generated by VSVΔ51GMCSF treatment. Surgical stress can induce immunosuppression or tumour growth facilitation. We are currently characterizing this period of surgical stress, and we are also aiming to overcome this stressed state by altering viral treatment regimens and route of administration.

The Immune Enhancing Effects of IL-7 on Human T Cells are Due to Activation of Stat Signaling but Not Repression of Cbl-b

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The E3 ubiquitin ligase Cbl-b plays a key role for anti-tumor immune responses, as cbl-b deficient mice are protected against different tumors and cbl-b deficient CD8 T cells efficiently reject tumors. We have recently shown that transient silencing of cbl-b in human T cells was able to reproduce the phenotype of cbl-b deficient murine T cells, validating cbl-b as target for cancer immunotherapy. Very recently, IL-7 has been described to control cbl-b expression and adjuvant IL-7 improved antitumor responses in murine models. We have therefore investigated whether IL-7 treatment of human T cells induces comparable biological effects as described for murine T cells. Cbl-b silencing or IL-7 treatment enhanced proliferation of TCR-stimulated CD4 and CD8 cells and induced increased production of IFN-γ, TNF-α and IL-2. However, IL-7 treatment of cbl-b silenced T cells induced similar effects, suggesting mechanistic independence from cbl-b. Accordingly, IL-7 treatment of human CD4 and CD8 T cells did not modulate cbl-b expression. We next tested the role of STAT factors, as IL-7 is a member of the common cytokine receptor γ-chain family (γc), which are known to signal via STAT transcription factors. Indeed, IL-7 induced STAT5 phosphorylation in human CD4 and CD8 T cells although with kinetic differences: in CD8 T cells STAT5 phosphorylation peaked earlier and declined more rapidly as compared to CD4 T cells. In agreement, the enhancement of cytokine production showed similar differences. IL-2, another member of the γc cytokine family, is clinically used as immune stimulant in cancer therapy. Thus, we have directly compared the effects of IL-2 and IL-7 on human immune cells. Like IL-7, IL-2 did not modulate cbl-b expression, but both cytokines induced STAT5 phosphorylation and enhanced proliferation and cytokine production. However, IL-7 failed to support the growth of human CD8 cells as efficiently as IL-2 did. Moreover, IL-2 but not IL-7 was able to activate human NK cells, thus suggesting that IL-7 will not have superior effects over IL-2 in clinically applied immune therapies against tumors. In conclusion, we show here that IL-7 does not modulate cbl-b expression in human T cells and therefore findings in the murine system using IL-7 might not be directly translatable into the clinic. In contrast, interventions targeting cbl-b have identical effects in murine and human T cells and thus seem to be a more promising strategy to enhance immune cell-mediated reactivity targeting tumors in vivo.

Ovarian Cancer Cells Ubiquitously Express HER-2 and can be Distinguished from Normal Ovary by Genetically Redirected T Cells

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Background: HER-2-specific T cells can be induced by vaccination or generated de novo by genetic engineering, however it remains uncertain to what extent T cell-based HER-2-directed immunotherapy can be utilized for the treatment of advanced ovarian cancer.
Objective: To validate HER-2 as a well-suited tumor antigen for widespread T cell-based adoptive immunotherapy of ovarian cancer.
Methods: HER-2 expression was first evaluated using immunohistochemical analysis (IHC) in 50 high-grade ovarian serous carcinomas. To determine the relative expression of HER-2 in ovarian cancer cell lines, patient tumor samples and normal ovarian surface epithelial cells (OSE), Q-PCR, FACS and western blot was performed. Human T cells were genetically engineered to express the C6.5 HER-2-specific chimeric immune receptor (CIR). HER-2-redirected T cells were tested for their capacity to recognize and kill HER-2 expressing tumors and OSE cells.
Results: IHC analysis showed HER-2 expression in 52% of primary OvCas; 26 cases had HER-2 expressed at one or more tumor sites while HER-2 was undetectable in 24 samples. However, Q-PCR, FACS and western blot analysis demonstrated HER-2 expression in all established ovarian tumors (13/13) and short-term cultured tumors (7/7). Consistent with these results, all tumor cells derived from primary ascites (24/24) and solid tumor (12/12) expressed HER-2, albeit at variable levels. Compared to tumor, all ($n = 4$) normal OSE expressed lower but detectable HER-2 levels. Genetically redirected T cells recognized and reacted against all ovarian cancer cell lines (14/14), primary ascites (5/5) and solid tumor (5/5) tested, however little or no reactivity was observed against normal OSE (1/4).
Conclusions: Our results show that IHC under represents the frequency of OvCas that express/overexpress HER-2 which may exclude patients with low HER-2 expressing tumors from receiving HER-2 targeted therapy. Utilizing more sensitive detection methods, we found that OvCas ubiquitously express HER-2, and generally at higher levels than normal ovary tissue. Importantly, all HER-2 expressing tumors are recognized by HER-2-redirected T cells and the latter are sensitive to even low levels of HER-2 expressed by OvCas. Importantly the CIR is able to distinguish recognition of ovarian cancer from normal targets, despite the fact that the normal cells do express HER-2 and therefore may minimize the potential for "off target" reactivity. These findings provide the rationale for the development of HER-2-redirected T cell-based immunotherapeutic approaches in women with ovarian carcinoma.

The Expression of Foxp3 in Tumor Tissues and Cancer Cell Lines of Epithelial Ovarian Cancer

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Objectives: The forkhead box protein 3 (Foxp3) transcription factor is a master control gene of the function of regulatory T cells. Recently, some publications have revealed that Foxp3 in cancer cells was expressed with high level and Foxp3 might be associated with carcinogenesis and cancer progression. On the contrary, some researchers have reported that Foxp3 was a tumor suppressor gene. The purpose of this study was to investigate the expression of Foxp3 in tumor tissues and cancer cell lines of epithelial ovarian cancer.

Materials and Methods: We obtained 21 tumor tissues from patients with epithelial ovarian cancer during initial operation and 4 different ovarian cancer cell lines, OV-CAR-3, SK-OV-3, SNU-8 and SNU-251, were selected. We used the fresh tumor tissues and the cell lines cultured for a period of 2 weeks. We isolated total RNAs using Micro-to-Midi total RNA purification system. Foxp3 mRNA was detected using real-time RT-PCR; PCR primer set for Foxp3 (SuperArray, USA), forward 5′-GGC ATCGTGATGGACTCCG-3′ and reverse 5′-GCTGGAAGGTG GACAGCGA-3′. Foxp3 expression is presented as a semiquantitative measurement obtained by calculating the intensity quotient for the gene and β-actin.

Results: The expression of Foxp3 mRNA was revealed in all tumor tissues and cancer cell lines. In 21 tumor tissues, median level of dCt was 6.92 (4.71 to 10.25). Comparing the result in normal ovarian tissues, relative expression level was high (median 2^{-} ddCt: 4.69). This expression also increased in tumor tissues of epithelial ovarian cancer comparing that in tissues of benign ovarian tumors. In 4 cancer cell lines, the expression of Foxp3 mRNA increased, especially in OV-CAR-3 cell lines.

Conclusion: Foxp3 mRNA was detected in epithelial ovarian cancer and the expression increased in tumor tissues and cancer cell lines comparing that of control. Therefore, Foxp3 could have a role in carcinogenesis or cancer progression. This result warrants further studies to understand the function of Foxp3 related to cancer immunity and carcinogenesis.

Extended Immunological Analysis of Two Phase I Clinical Trials of MVA-BN®-HER2 in HER-2 Overexpressing Metastatic Breast Cancer Patients

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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 antitumor activity against HER-2 expressing tumors (Mandl et al. Abstract ISBTC 2010). Previous immunological evaluation of MVA-BN®-HER2 treated patient samples revealed that treatment was able to break tolerance against HER-2 in a metastatic setting, inducing a humoral and/or T cell response in greater than 66% of the patients. Specifically, anti-HER-2 antibodies were detected in 52% of patients tested and T cell responses were boosted in 63% of patients. Here we report on extended immunological analysis of cryopreserved PBMCs and sera from patients receiving MVA-BN®-HER2.

The MVA-BN® viral vector activated innate immune responses, potentially propagating antitumor responses. This was noted by an increase in natural killer cytolytic activity in 50% of evaluated patients as well as an expansion of gamma-delta T cells, a population having direct antitumor cytotoxic functions. Adaptive cellular immune responses were also augmented post treatment. MVA-BN®-HER2 vaccination elevated CD8 effector T cell levels, resulting in an increased CD8 effector to Treg ratio. In contrast, high levels of CD4/CD8 double positive T cell levels, a possible regulatory population, were detected in low responding patients.

Humoral immune responses were further analyzed in two new assays: (1) a flow cytometry based titer assay to characterize anti-HER-2 antibody binding to HER-2 expressing cells, and (2) a peptide array comprised of 7590 peptides derived from 46 breast cancer tumor associated antigens (TAA)s including HER-2. In patients treated with MVA-BN®-HER2, qualitatively different anti-HER-2 antibody responses were measured by these assays as compared to previous ELISAs. In addition, 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.

Taken together, these data support that MVA-BN®-HER2 treatment is a potent activator of both the innate and adaptive arms of the immune response. The broadening of immune responses to non-HER-2 TAAs suggests that the MVA-BN®-HER2-mediated immune activation resulted in anti-tumor activity.

Clinical and Immunological Analysis of a Phase I Trial Evaluating MVA-BN®-PRO in patients with Non-metastatic Castration Resistant Prostate Cancer (CRPC)

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MVA-BN®-PRO is a candidate prostate cancer immunotherapy product comprised of a highly attenuated non-replicating vaccinia virus, MVA-BN®, engineered to encode prostate specific antigen (PSA) and prostate acid phosphatase (PAP) proteins. Pre-clinical studies in mouse tumor models have demonstrated vaccine-mediated induction of anti-PSA and PAP specific immune responses as well as anti-tumor activity (Rountree et al. Abstract ISBTC 2010). A Phase I trial was designed as an open-label multicenter dosing evaluation of MVA-BN®-PRO in patients with non-metastatic castration resistant prostate cancer (CRPC). Patients were immunized subcutaneously with 1, 2 or 4 doses of the study drug three times at monthly intervals, followed by re-treatment with an additional 3 vaccinations at the same dose. Patients with stable disease were offered a 1-year extended treatment phase. To date, there have been no dose-limiting toxicities (DLT) identified and no drug-related severity of adverse reactions (SAE) noted in the 24 patients enrolled. Of the 21 patients having successfully completed treatment, 7 patients demonstrated stable serum PSA levels and continued treatment, receiving up to 12 additional administrations at the same dose and schedule.

Preliminary immune evaluation of the 21 patients completing treatment with MVA-BN®-PRO revealed that 15 patients developed vaccine-induced humoral and/or cellular immune activity to PSA and/or PAP. Specifically, PSA and/or PAP specific antibodies were detected in 7 out of 21 patients completing treatment. And although modest, T cell responses were boosted post-treatment in 12 out of 21 patients. Moreover, continued treatment (up to 18 total vaccinations) did not adversely impact immune responses as measured by T cell proliferation and IFN-γ secretion by ELISPOT.

In 5 out of 6 evaluable patients continuing treatment, the response to MVA was boosted, and in 4 out of 6 evaluable patients, transgene responses increased either in magnitude and or in breadth. Furthermore, continued treatment did not impact regulatory T cell (Treg) levels, which predominantly remained stable with a few patients showing reduced Treg levels upon continued vaccination.
Overall, this analysis indicates that MVA-BN®-PRO can break tolerance against PSA and/or PAP in cancer patients. It also suggests that transgene-specific immunity can be achieved in patients despite the pre-existence or co-induction of strong anti-MVA responses. Based on this interim analysis and contingent upon complete and confirmatory results, it appears that MVA-BN®-PRO is a biologically active treatment for patients with non-metastatic CRPC.

In Vivo Modeling and Detection of Ovarian Cancer Vascular Marker TEM1

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Rationale: Epithelial ovarian cancer (EOC) remains the most deadly cancer without efficient detection and eradication methods. TEM1 is overexpressed specifically in the vasculature of various tumors and has been implicated in promoting adhesion, invasion and metastasis. Since EOC responds well to vascular-targeted therapy, we hypothesize that TEM1 is a tumor vascular marker with diagnostic and therapeutic potential.
Specific Aims: (1) To characterize TEM1 expression in normal tissues and EOC samples; (2) to establish huTEM1 murine tumor vasculature model; (3) to develop a TEM1-specific PET imaging strategy.
Methods: qPCR and IHC are used to characterize huTEM1 expression in normal and EOC samples. Luciferase positive huTEM1 expressing murine endothelial cell (huTEM1+ EC) lines were established and injected alone or with tumor cells onto nude mice and monitored by bioluminescent imaging.
An antiTEM1 Ab, MORAb004, was radiolabeled with 124I and injected venously. PET images were acquired at various time points to visualize hTEM1+ECs in vivo and corresponding biodistribution studies were performed.
Results: (1) High TEM1 mRNA level correlates with poor outcome in 2 cohorts of EOC patients. (2) Positive TEM1 staining was observed in most EOC tissues studied, while no positive staining was seen in controls. (3) TEM1 expression in huTEM1+ ECs was confirmed by qPCR, western and FACS analysis. (4) huTEM1+ and control ECs can be detected in nude mice 5 weeks p.i.5) [124I]MORAb004 has been successfully labeled in high yield and without loss of immunoreactivity. (6) PET images revealed specific retention of [124I]MORAb004 in huTEM1+ xenografts and no discernible uptake in control of the same animal. The radioactivity in TEM1+ tumor lasted >6 days p.i. (7) Ex vivo biodistribution study at 48 hours p.i. revealed hTEM1+ xenograft to nontarget ratios (T/NT) upwards of 16, 84, and 9 for blood, muscle, and control xenograft, respectively.
Conclusions: (1) Our data suggest that TEM1 is a rational diagnostic and therapeutic target for EOC. (2) We developed PET imaging strategy to visualize huTEM1+ cells in mouse model. (3) Our murine vascular model allows quantitative and specific monitoring of ECs by optical and PET imaging, therefore serves as an unprecedented platform for studying the function of tumor vascular markers, as well as testing new diagnostics and therapeutic agents against tumor vasculature in vivo. (4) Further studies will evaluate TEM1 as early detection marker and prognostic factor for EOC.

Analysis of Intra-Tumor Immunological Changes Induced by Immunization with Two-Component Messenger RNA-Based Anti-Cancer Vaccines

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Two-component tumor mRNA-based vaccines exhibit two principle activities: antigen expression and concomitant immune stimulation. Thus, innate as well as adaptive immune responses are induced. Intradermal vaccination of tumor-bearing mice with the two-component mRNA vaccine mediates a strong anti-tumor response under therapeutic conditions. Depletion experiments demonstrate the requirement of CD8 T cells for the anti-tumor effect and the necessity of CD4 T cell help for the induction of antigen-specific CTLs. FACS analysis of tumor tissue revealed increased infiltration of activated CD8 T cells and their prolonged persistence at the tumor site in response to vaccination. Moreover, therapeutic vaccination inhibited a tumor-induced increase of myeloid derived suppressor cells (MDSCs) in the spleen and at the tumor site.
To further elucidate the mechanism of our mRNA-based anti-cancer vaccine, tumor-bearing mice were vaccinated repeatedly and tumors were removed at different time points. Microarray analysis of total mRNA extracted from removed tumors revealed clear differences between vaccinated and control mice. Already after two vaccinations, before the effect on tumor size became visible, a wide variety of immune response related genes was upregulated in vaccinated mice. A large proportion of these genes is associated with activation and cytotoxicity of NK and T cells, Th1 polarization or chemotaxis.
Our findings conclusively demonstrate the comprehensive nature of the immune response induced by our mRNA based vaccines and the variety of pathways involved in the anti-tumor effect. Better understanding of the mode of action allows further improvement of our vaccine approach and the selection of potential targets for combination therapies. Additionally, the approach opens new possibilities for targeted monitoring of induced immune responses.

Targeting of Mortalin-Mutant p53 Interactions by Mortalin shRNA Leads to Selective Apoptotic Death of Human Hepatocellular Carcinoma

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Restoration of deregulated apoptotic pathway is one of the main strategies for cancer therapeutics. Mortalin/mitochondria heat shock protein 70 (mtHSP70) is a stress protein that is overexpressed in cancer cells and tissues, which has been proposed to have a role in human carcinogenesis. It was previously shown that mortalin interacts with wild type tumor suppressor protein p53 resulting in abrogation of its transcriptional activation and control of centrosome duplication functions, both tightly related to cancer development. Normal human fibroblasts were shown to lack mortalin-wild type p53 interactions. It was also identified as a marker for hepatocellular carcinoma (HCC) metastasis and recurrence by proteomics analysis of matched tumor and non-tumor tissues. In this study, we examined mortalin expression in 100 HCC patients and found that its upregulation has strong correlation with tumor stage and microsatellite formation. In order to validate the critical role of mortalin in HCC development and progression, we recruited its shRNA in eight HCC-derived cell lines varying in p53 status (loss/mutant p53/wild type). Mortalin-shRNA caused apoptosis in most, but not all, HCC cell lines. By examination of mortalin-p53 interactions and molecular pathway for apoptosis, we found that induction of apoptosis by mortalin-shRNA depended on occurrence of mortalin-mutant p53 interactions; the cell lines that lacked these interactions escaped apoptosis. Most importantly, mortalin-p53 interactions were induced by

cellular stress by chemotherapeutic drug (cisplatin) following which the cells were sensitized to mortalin shRNA induced apoptosis. Based on the data, a model for mortalin and p53 interactions in cancer (physiologically a stressed condition) cells and use of mortalin-shRNA for selective killing of cancer cells is documented. Since p53 mutations are found in more than 80% of the tumors, mortalin shRNA is proposed as an effective cancer cell specific therapeutic tool.

Immunomodulation of T Cell-Mediated Activity by Cancer Stem Cells Isolated from Human Glioblastoma

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The main objectives of our study was to assess whether cancer stem cells (CSCs) isolated from glioblastoma multiforme (GBM) can represent a target for immunotherapeutic treatment for GBM patients. We have previously reported that GBM CSC have a low immunogenic profile, eliciting mostly TH2-mediated responses in autologous settings and inhibit allogeneic T cell proliferation compared to their FBS-cultured non-CSC (FBS tumor cells) pairs (Di Tomaso et al, 2010). This immune-inhibitory activity mediated by GBM CSCs was independent on the secretion of TGFβ-1 or 2, that were preferentially down-modulated in CSC lines, nor on IL-10 and IL-13 that were undetectable in the supernatant of these cell lines. We found that both CSCs and FBS tumor cells expressed immune response inhibitory molecules, such as CTLA-4, PD-1 and PDL-1. Furthermore, a differential gene signature that was confirmed at the protein level for some immunological-related molecules was also found between CSC and FBS lines. This latter analysis provided information of some candidate molecules associated with CSCs that we are functionally investigating. A candidate negative immunoregulatory molecule is represented by the indoleamine 2,3-dioxygenase (IDO), a molecule implicated in the generation of immune tolerance. By RT-PCR we detected the expression of the mRNA of this molecule in both CSCs and FBS tumor cell lines with high increase following IFN-γ treatment of the cells. We assessed the functional activity of IDO determining by a colorimetric assay IDO-mediated tryptophan catabolism in culture supernatants, and, notably, in some cases a higher activity was associated with IFN-γ treated CSCs but not with their FBS tumor counterparts. Interestingly, IDO-mediated activity was inhibited by treatment of these cells with the specific inhibitor 1-Methyl Tryptophane (1-MT). Thus, these results indicate that IDO can represent a candidate molecule mediating immunosuppressive functions of GBM CSCs. We are currently carrying out experiments to demonstrate whether the blocking of IDO in GBM cells can increase the efficiency in raising CSC-specific T cell responses. Altogether, these results indicate a lower immunogenicity and higher suppressive activity of GBM CSC compared to autologous FBS lines and the need to identify immunomodulatory agents that can efficiently restore the expression of immunogenic molecules on CSCs.

Anti-Tumor Efficacy of MVA-BN®-HER2 is Mediated by HER-2-specific Th1-immunity and the Control of Tumor-Induced Immunosuppression

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MVA-BN®-HER2 is a candidate immunotherapy product for the treatment of HER-2-positive breast cancer that has shown biological activity in Phase I clinical trials (Legrand et al. Abstract iSBTc 2010). MVA-BN®-HER2 is derived from the highly attenuated vector MVA-BN® and encodes a modified form of the HER-2 protein, referred to as HER2. HER2 comprises of the extracellular domains of HER-2 and has been modified to encode two universal T-helper cell epitopes from tetanus toxin. We have previously demonstrated in pre-clinical models that both adaptive and innate mechanisms contribute to the anti-tumor activity of MVA-BN®-HER2. Here we further explore the adaptive arm of immune responses elicited by treatment with MVA-BN®-HER2 and present insight into the mechanism of action of MVA-BN®-HER2-induced anti-tumor activity in a murine model of experimental pulmonary metastasis (CT26-HER-2). Treatment with MVA-BN®-HER2 induced potent Th1-dominated anti-HER-2 antibody and T cell responses in mice. More importantly, immunotherapy consisting of a single dose of MVA-BN®-HER2 exerted significant anti-tumor efficacy which was accompanied by a high density of CD8+ T cells in the tumor. Phenotypic analysis of the tumor infiltrating lymphocytes (TIL) revealed a population of highly activated, HER-2-specific, CD8+CD11c+ T cells. Notably, MVA-BN®-HER2 treatment was able to override the immunosuppressive responses generated by the growing tumor by reducing the frequency of Treg cells, thus resulting in a significant improvement in the ratio of CD4 and CD8 T effector cells to Treg cells in the tumor. In contrast, treatment of tumor-bearing mice with HER2 protein formulated in Complete Freud's Adjuvant (CFA) induced a strong Th2-biased immune response to HER-2. However, this did not lead to significant infiltration of the tumor by CD8+ T cells, the reduction of the frequency of Treg cells, or anti-tumor efficacy. The respective roles of CD8, CD4, or CD25 positive T cells in the anti-tumor activity of MVA-BN®-HER2 and in this tumor model per se were further investigated by in vivo depletion of specific T cell populations. Depletion of CD8+ cells strongly reduced the anti-tumor activity of MVA-BN®-HER2, confirming that CD8 T cells are required for protection. Depletion of CD4+ or CD25+ cells confirmed that tumor-induced Treg cells promote tumor growth. Taken together, our data demonstrate that MVA-BN®-HER2 controls tumor growth through a combination of the induction of Th1-biased HER-2-specific immune responses and the control of tumor-induced Treg cells.

Allogeneic Transplantation and Tumor-Specific Vaccination Promote Infiltration and Eradication of Advanced Prostate Cancer

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Central and peripheral mechanisms of tolerance hinder protective immunity against tumor. We recently reported that non myeloablative allogeneic transplantation and tumor-specific vaccination overcome tumor-specific tolerance allowing acute rejection of spontaneous prostate cancer and prolonging disease-free survival (Hess Michelini, Cancer Res. 2010). This was distinctively due to the concomitance of high affinity tumor- and minor histocompatibility (H) antigen-specific T cells. To envisage clinical translation of this strategy we evaluated its efficacy in the context of inevitable multiple minor H antigen disparities generally observed between donor/recipient pairs. In spite of donor T cells infiltrating normal tissues and of clinical signs of graft versus host responses, prostate cancer rejection was also found in this setting. Rather than providing reciprocal T cell help at the time of priming in peripheral lymphoid organs, concomitant minor H antigen-and tumor specific T cell responses allowed potent tumor infiltration. Confocal images showed that in recipients of minor H antigen mismatched lymphocytes CD3+ and CD8+ T cells were found juxtaposed to prostate epithelial cells with higher expression of MHC class I molecules and of minor H antigens. This was paralleled by the enrichment of IFN-γ, perforin and granzyme B mRNA, and

caspase 3+ cells, which reflected tumor rejection. Together our data indicate that non-myeloablative allotransplantation followed by tumor-specific vaccination promotes potent graft versus tumor responses by enabling massive tumor infiltration and leading to the rejection of advanced prostate cancer. Thus, this combined strategy should be attempted for the cure of patients with solid tumors.

Ipilimumab Monotherapy in Melanoma Patients with Brain Metastases-Update of a Phase II Study

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We present results from a Phase II trial (CA184-042) to assess the anti-CTLA-4 antibody ipilimumab (IPI) safety and activity in advanced melanoma patients (pts) with brain metastases (mets). Pts were corticosteroid-free (Arm A; n = 51) or required a stable dose of corticosteroid for symptoms or edema (Arm B; n = 21). All pts had measurable brain mets with at least one lesion 0.5 to 3 cm or $\geq$ 2 lesions 0.3 to 3 cm in diameter. Prior treatment of non-index lesions with whole brain (WBRT) and/or stereotactic radiotherapy (SRT) was allowed. IPI was given IV, 10 mg/kg, every 3wks for 4 doses; responding or stable pts were to receive maintenance IPI 10 mg/kg, every 12 weeks. Tumors were assessed (modified WHO criteria) every 6 weeks; objective response (OR) and stable disease (SD) as best response required confirmation at 4 weeks. In Arm A, 4 had prior SRT and 17 prior WBRT (no pt had both); in Arm B, 5 had prior WBRT. Considering all lesions (global response of brain and extracranial tumor) at Wk12, 5/51 pts in Arm A had a partial response (PR) and 4/51 pts had SD [global disease control rate (DCR) 17.6% (95% CI 8.4-30.9)]. For brain alone, 8 pts in Arm A had PR and 4 SD [DCR 23.5% (95% CI 12.8-37.5)]. Starting from the Wk12 landmark, response durations ranged from 3 to 17+ months and duration of SD from 1 to 11+ months. Median progression-free survival (PFS) was 1.4 months (95% CI 1.2-2.5). In Arm B, pts achieved global PR (n = 1), PR in brain (n = 1), SD in brain (n = 1) and PR (n = 1) in non-CNS tumor with a median PFS of 1.2 months (95% CI 1.2-1.3). CNS AEs were similar regardless of steroid use in pts in Arm B: CNS AEs of any grade occurred in 39/51 pts (76.5%) (16 Grade 3 to 4; 31.4%) in Arm A, and in 14/21 pts (66.7%) (7 Grade 3 to 4; 33.3%) in Arm B. The most common events (> 10% pts) were headache, dizziness, and seizures; all attributed to tumor. There was no association between brain edema or cerebral hemorrhage and OR. The frequency and distribution of autoimmune AEs outside of the CNS were similar to all other experience with this dose and schedule of IPI. In conclusion, IPI treatment appears to be similarly active against advanced melanoma brain mets and non-CNS mets. IPI was well tolerated with no unique toxicities in melanoma pts with brain mets; pts on low doses of corticosteroids may also benefit from treatment with IPI.

Clinical and Immunological Response in Castration-Resistant Prostate Cancer Patients Treated with an Epidermal Growth Factor (EGF)-Based Vaccine

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EGF is a potent growth factor that is believed to enhance the proliferation of cancer cells. Recently, it was reported that 100% of castration-refractory prostate tumors overexpress the Epidermal Growth Factor Receptor (EGFR). We have developed a new active specific immunotherapy based on EGF deprivation. In this study we characterize the humoral and clinical response in advanced prostate cancer patients included in an ongoing Phase II clinical trial. Castration-resistant metastatic prostate cancer patients were randomized to receive the EGF vaccine or best supportive care. At least two vaccinations were given before the first line of chemotherapy treatment (mitoxantrone), with subsequent monthly vaccination after concluding chemotherapy. The primary end points were immunogenicity and safety.

A subset of patients was studied for immunological response. With this schedule of treatment, anti-EGF IgG antibody titers were more than 20 times higher than those previously obtained, without any increase in adverse events. Ninety-six percent of vaccinated patients developed a good antibody response (GAR), while none of the controls did. Notably, 50% of patients were classified as very good antibody responders (SGAR). The global immune response was not affected with chemotherapy treatment. On the other hand, 41% of evaluated patients showed an immunodominant antibody response against the central region on the EGF molecule (loop B). A trend to better survival was found for vaccinated patients that showed an immunodominance by the loop B. Concerning to clinical response, 104 patients were evaluated. There was a trend to an increased survival for vaccinated patients (median = 17.1 mo) compared with controls (median = 10.13 mo), without a significant change in PSA levels. The survival advantage for vaccinated patients compared with controls was statistically significant in the subgroup of patients with more undifferentiated tumors. In summary, this study has shown that combination of EGF vaccination at high dose, with chemotherapy is feasible and well tolerated in castration-resistant prostate cancer patients. Most patients developed high anti-EGF antibody titers and immunodominance toward loop B seems to be associated with clinical benefit. In poor prognostic patients, vaccination was associated with increased survival.

The High-Dose Aldesleukin (HD IL-2) "Select" Trial in Patients with Metastatic Renal Cell Carcinoma (mRCC)

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Background: HD IL-2 received FDA approval for mRCC in 1992, producing a 14% major response (CR + PR) rate and durable remissions in Phase II trials. Retrospective studies suggested that tumor features could predict for benefit (Atkins et al, Clin Cancer Res 2003). The Cytokine Working Group conducted this prospective trial to identify patients (pts) likely to respond to HD IL-2.

Methods: In this multicenter, prospective study pts with histologically confirmed mRCC, ECOG PS 0-1 and adequate organ function received HD IL-2. The primary endpoint of the study was to determine if the major response rate (RR) of pts with "good" predictive features was significantly higher than a historical, unselected population. All pts were consented to provide archived tumor tissue that would be used for pathology risk classification.

Results: One hundred twenty eligible pts enrolled between November 2007 and July 2009. Seventy-two percent had ECOG PS 0, 71% were MSKCC intermediate risk, 96% had clear cell (cc) RCC and 99% had prior nephrectomy. There were 2 treatment-related deaths. At the time of this analysis the investigator assessed RR was 28% (34/120) (7 CR, 27 PR) and was significantly greater than the historical RR (95% CI = 21%-37%, $P = 0.0016$).

TABLE 1. Response by Pathology Characteristics

Histology Risk Group	(RR%) (95% CI)	P
Good (n = 11)	36 (14%-34%)	0.61
Intermediate (n = 84)	26 (17%-37%)	
Poor (n = 24)	33 (16%-55%)	
CA-9 score		
High (> 85% n = 77)	23 (14%-34%)	0.13
Low (85% n = 39)	38 (23%-55%)	
Combined score		
Good (n = 74)	24 (15%-36%)	0.67
Poor (n = 42)	36 (22%-52%)	

The median PFS was 4.2 months (mo) and 17 responses are ongoing (range 6 to 41+ mo). The RR for pts with ccRCC was 30% (35/115) (95% CI = 21%-39%, $P = 0.0004$). Response to IL-2 was seen in pts in all MSKCC risk classifications, but was not seen in pts with non-cc histology (5 pts) or high UCLA SANI (survival after nephrectomy and immunotherapy) score (8 pts). Clear cell histologic classification and high CA-9 staining failed to further enrich for response to IL-2 (Table 1).

Conclusions: The RR to HD IL-2 in this trial was significantly better than the historical experience. Analysis of tumor based predictive markers through central pathology review was unable to improve the selection criteria for HD IL-2. Efforts to validate other proposed biomarkers (eg, CA-9 SNPs, B7H1 expression and serum VEGF) predictive of response to IL-2 are ongoing and will be presented at the meeting.

A Paradigm Shift in Ovarian Cancer Immunotherapy: Breaking Immune Tolerance Redirecting T-Helper Differentiation with Cancer/Testis Antigen Vaccination

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Ovarian cancer (OC) is a fatal malignancy that and accounts for the majority of gynecologic tumor-related deaths. Cancer vaccines are considered less toxic and more specific than standard treatments. OC expresses a number of potential antigens, but immune responses to OC are hampered by immune evasion mechanisms, as the recruitment of suppressive T cells (T-reg) and the inhibition of effector IL-17 producing T cells (Th-17). Therefore, a strategy to overcome tumor-induced immune suppression could save patients' lives. We have shown that CpG -adjuvated SP17 vaccination affords long-term protection form tumor growth and spread in the C57BL/6-ID8 murine model of OC. Here we elucidate the mechanism of action of our vaccine, by a complete phenotypic characterization of the T-lymphocyte subsets in tumor challenged mice undergoing different vaccinations, performed by multiple techniques, namely flow-cytometry, multiplex ELISA and RT-PCR.

We show that the contemporary administration of SP17 protein and CpG produced a dramatic skewing in T-lymphocytes subpopulation frequencies. Specifically, we detected a decrease in immune-suppressor T-reg and Th-2 type cells and a contemporary increase in activator Th-17 and Th-1 type cells frequency in SP17 + CpG vaccinated animals only. Importantly, the striking stimulation of Th-17/Th-1 response correlated with the activation of INF-g producing CD8+ cytotoxic lymphocytes, but did not result in any toxic effect, such as autoimmune diseases.

The ability of our vaccine to reprogram T cells differentiation from T-reg to Th-17 is highly relevant for translation in the clinical practice, since in OC lesions have been shown to recruit T-reg cells, while Th-17 activation is associated with better prognosis.

In conclusion, or vaccine is likely to be successfully used together with standard treatments for the cure of primary as well as relapsed OC, preventing tumor growth and dissemination in vivo.

Characterizing Host Responsiveness to Interferon by Ex Vivo Induction of Interferon-Responsive mRNAs

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Interferons (IFN) can be effective clinically for patients with infectious diseases (hepatitis B and C), autoimmune disorders (multiple sclerosis) and malignant neoplasms (leukemia, lymphoma, melanoma). However, there remains a need for assessing the likelihood that a given individual will respond to IFN administration and to assess pharmacodynamics of response. In the present study, triplicate aliquots of 0.06 mL each of heparinized whole blood were stimulated with IFNs (INF alpha-2b, INF beta-1a, INF gamma) or solvent control for only 2 hours, then various IFN-responsive mRNAs were quantified by our high throughput assay. (Mitsuhashi et al. *Clin Chem*. 200652:634–642). Significant induction was identified for CCL chemokine-8, CXCL chemokine-9, 10, 11, tumor necrosis factor superfamily (TNFSF)-10, guanylate binding protein 1 (GBP1), XIAP associated factor 1 (XAF1), suppressor of cytokine signaling 1 (SOCS1), ISG15 ubiquitin-like modifier (ISG15, G1P2), bone marrow stromal cell antigen 2 (BST2), and interferon regulatory factor 7 (IRF7), whereas the levels of TNFSF5, interleukin (IL)-8, and IL23 were decreased. The increase or decrease of these mRNAs happened rapidly, and reached a plateau after 2 to 4 hours. The reaction was dose dependent from 10,000 to 100,000 units/mL of IFN alpha-2b. To confirm the ex vivo results, blood was drawn into PAXgene tubes before and after IFN-alpha 2b under an IRB-approved protocol. Total RNA was extracted from nucleated cells and used to measure mRNAs using the same method as the ex vivo assay. As a result, the increase and decrease of above mentioned mRNAs were confirmed in this in vivo assay. Within 4 hours after IFN administration, more than 32 folds induction of TNFSF10, SOCS1, CXCL10, CXCL11, G1P2, and GBP1 mRNA were identified. Moreover, the levels of these mRNA markers changed during clinical course, suggesting that the assay can be used to monitor host responsiveness to IFN and/or other therapies and immunomodulators. The throughput seamless process of the assay allows us to manipulate many samples simultaneously, and small variation among triplicate samples provides sensitivity to detect 50% change with statistical significance for each mRNA.

Pegylated Arginase I: A Potential Therapeutic Approach in T-ALL

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Adult patients with acute lymphoblastic T cell leukemia (T-ALL) have a very poor prognosis and few effective therapeutic options. Therefore, novel therapies that increase the efficacy of the treatments and that prolong T-ALL patient survival are needed. Malignant T cells require high concentrations of nutrients to sustain their increased rate of proliferation. In this study, we determined whether L-Arginine depletion by the pegylated form of the L-Arginine-metabolizing-enzyme arginase I (peg-Arg I) impairs

the proliferation of malignant T cells. Our results show that peg-Arg I depleted L-Arginine levels in vitro and in vivo. In addition, treatment of malignant T cell lines with peg-Arg I significantly impaired their proliferation, which correlated with a decreased progression into the cell cycle, followed by the induction of apoptosis. Furthermore, peg-Arg I impaired the expression of cyclin D3, a fundamental protein in T-ALL proliferation, through a global arrest in protein synthesis. Injection of peg-Arg I plus chemotherapy agent Cytarabine prolonged survival in mice-bearing T-ALL tumors. This anti-tumoral effect correlated with an inhibition of T-ALL proliferation in vivo, a decreased expression of cyclin D3, and T-ALL apoptosis. The results suggest the potential benefit of L-Arginine depletion by peg-Arg I in the treatment of T cell malignancies.

Comparison of GMP and Non-GMP Enzymes for the Isolation of Tumor Cells and Tumor-Infiltrating Lymphocytes for the Immunotherapy of Melanoma

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Historically tumors were processed using non-GMP reagents to isolate tumor cells for vaccines or tumor-infiltrating lymphocytes (TIL) cultures. Encouraged by the FDA, we obtained GMP enzymes and evaluated our recovery of viable tumor cells from tumor specimens processed using these enzymes and compared this to our previous experience using non-GMP enzymes. We processed 33 freshly resected consecutive melanoma specimens using GMP enzymes. We then compared cell yields against those obtained using non-GMP enzymes from 28 consecutive melanoma specimens received prior to January 8th, 2009. Statistical Methods: After log10-transformation of both variables, cell yield visually appeared as a linear function of tumor weight for both types of enzyme. The log10 cell yield was linearly regressed against log10 tumor weight. The regression model included separate intercepts and slopes for both types of enzyme. 95% confidence intervals were calculated for each model parameter. To compare the intercepts and slopes for each type of enzyme, *P*-values were calculated using contrasts and 90% confidence intervals were calculated to assess equivalence. To assess the suitability of the linear model, two methods were used. First, a quadratic term was added to the linear regression model. Second, local regression (loess) was fit using a local linear approximation. The smoothing parameter was chosen by visually assessing smoothness and evaluating a plot of bias-corrected AIC vs. smoothing parameter. R-squared was calculated for the model after verifying that the linear model was adequate. Results: Log10-transformation of both cell yield and tumor weight resulted in the linear relationship with the intercepts and slopes for each type of enzyme being similar. Additionally, the generation of melanoma cell lines was similar for preparations isolated using non-GMP (16/28 57.1%) or GMP enzymes (17/33 51.5%). While not directly compared, outgrowth of TIL (13/19 68.4%) was comparable to our previous experience. Together, these data confirm that GMP enzymes can be used to generate tumor and TIL for cancer immunotherapy.

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Effect of IgE on Cancer

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IgE -mediated activation of basphils and mast cells is central to the allergic response. The cytosolic free calcium elevation(Ca++) that follows the activation through high-affinity IgE receptor is generally considered a key element among the signaling events responsible for secretion from these cells. IgE binding to mast cells via high affinity receptor is simply a passive sensitization step prior to activation by receptor aggregation. Recent studies have shown that even monomeric IgE can induce its own receptor up-regulation on mast cell. Recent studies provided evidence that Fc ERI dependent activation of mast cells is regulated by alternative signaling pathway, it activates (PLC8) lending to an increase in cytosolic calcium. IL-2 production requires an increase in Ca++. This production appears to be the critical step requiring transmembrane calcium flux. Some results indicate that IL-2 production required Ca++ in the early stage. The induction of IL-2 receptor expression and IL-2. Production may involve different activation signals. An increase in cytosolic free calcium concentration is one of the signals involved in cellular activation nitrogen stimulation leads to rises in mean intracellular calcium level. Hata D et al, proposed that cross-linking of the high affinity IgE receptor (F cepsilon RI) on mast cells induce secretion of cytokines IL-2 through transcriptional activation of cytokine genes. The majority of preclinical data gathered lin therapeutic model of Il-2 against several syngeneic animal tumors.

Designing a New Chimeric Antigen Receptor for the Tumor Antigen ALK

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The protein ALK (CD246) is receptor tyrosine kinase expressed during embryonic development, primarily in the nervous system. ALK was originally described as an intracellular fusion protein with nucleophosmin in analpastic large cell lymphoma (ALCL). In neuroblastoma, ALK mutations have been linked to familial inheritance of the disease. Unlike hematological malignancies, full-length ALK is re-expressed on the cell surface of pediatric solid tumors and contains activating mutations. Work in the Wellstein lab has identified the ALK ligand binding domain using a phage display methodology to block the binding of pleiotropin to ALK. We used the single chain scFV that was generated against this region of ALK as the starting point for the construction of a chimeric antigen receptor that featured the single chain Fv fragment linked to CAR transmembrane and signaling domains, or which also included the CH2CH3 regions of IgG, as a means to extend the scFV-derived sequences further away from the surface of transduced T cells. Preliminary data demonstrates that the extended CH2CH3 conformation is active against two neuroblastoma cell lines, while the single Fv-expressing construct has greatly diminished activity. In further work we propose to generate higher affinity CAR constructs and to develop these as potential agents for the adoptive immunotherapy of tumors expressing surface ALK.

Ipilimumab Improves Overall Survival in Previously Treated, Advanced Melanoma Patients with Good and Poor Prognostic Factors

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Ipilimumab is a fully human, monoclonal antibody that blocks cytotoxic T-lymphocyte antigen-4 to potentiate an antitumor T cell response. In a phase III, randomized controlled trial (MDX010-20), ipilimumab has demonstrated a statistically significant improvement in overall survival (OS) in previously treated, advanced melanoma patients. In this trial, randomized patients included those with M1c disease and elevated baseline levels of serum lactate dehydrogenase

TABLE 1.

Overall Survival (OS)	Ipilimumab + gp100 (n = 403)	Ipilimumab Alone (n = 137)	gp100 Alone (n = 136)
All patients			
Median OS (mo) (95% CI)	10.0 (8.5-11.5)	10.1 (8.0-13.8)	6.4 (5.5-8.7)
Hazard ratio vs. gp100	0.68 (0.55-0.85)	0.66 (0.51-0.87)	—
Log-rank *P* value vs. gp100	0.0004	0.0026	—
M0, M1a, M1b			
Median OS (mo) (95% CI)	15.0 (12.7-20.2)	19.9 (10.1-NR)	9.8 (6.3-11.1)
Hazard ratio vs. gp100	0.57 (0.38-0.87)	0.47 (0.27-0.82)	—
Log-rank *P* value vs. gp100	0.0079	0.0069	—
M1c			
Median OS (mo) (95% CI)	7.8 (6.5-9.6)	8.5 (5.8-11.2)	5.9 (4.7-7.5)
Hazard ratio vs. gp100	0.74 (0.58-0.95)	0.72 (0.53-0.97)	—
Log-rank *P* value vs. gp100	0.0187	0.0325	—
LDH ≤ ULN			
Median OS (mo) (95% CI)	14.2 (11.8-16.3)	16.5 (12.3-21.6)	9.7 (8.3-10.8)
Hazard ratio vs. gp100	0.70 (0.53-0.93)	0.56 (0.39-0.81)	—
Log-rank *P* value vs. gp100	0.0127	0.0016	—
LDH > ULN			
Median OS (mo) (95% CI)	4.8 (3.5-5.6)	4.9 (3.2-6.0)	4.0 (3.0-5.0)
Hazard ratio vs. gp100	0.71 (0.51-0.98)	0.76 (0.51-1.1)	—
Log-rank *P* value vs. gp100	0.0388	0.1667	—

CI indicates confidence interval; NR, not reached; ULN, upper limit of normal.

(LDH), which are associated with a very poor survival outcome in advanced melanoma.

In MDX010-20, 676 HLA-A*0201-positive patients with previously treated, unresectable stage III or IV melanoma were randomized 3:1:1 to ipilimumab plus a gp100 peptide vaccine (n = 403), ipilimumab alone (n = 137), or gp100 alone (n = 136). Ipilimumab at 3 mg/kg and/or gp100 were administered every 3 weeks for up to four treatments (induction). The primary endpoint was a comparison of OS between the combination and gp100 monotherapy groups. Pre-specified categories for subgroup comparisons of OS included M-stage (M0, M1a or M1b versus M1c) and baseline LDH levels [≤upper limit of normal (ULN) versus > ULN].

Ipilimumab, with or without gp100, resulted in a statistically significant improvement in OS compared with gp100 alone (Table). There was no difference in OS between the two ipilimumab groups (hazard ratio = 1.04; $P = 0.7575$). Subgroup analyses suggest that ipilimumab confers a survival advantage to previously treated patients with advanced melanoma who have good and poor prognostic factors (Table 1), although smaller patient numbers in certain subgroups, that is, ipilimumab monotherapy in subgroup LDH > ULN, may have limited the analyses.

Subcloning and Antiviral Phenotype Segregation in Pancreatic Cancer Cell Line Subpopulations

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Pancreatic adenocarcinoma (PDAC) remains a leading cause of cancer mortality for which novel gene therapy approaches relying on tumor-tropic adenoviruses are being tested.We published that HPAFI cell line is characterised by having an endogenous expression of interferon stimulated genes (ISGs) testified by the expression of the marker Myxovirus resistence A (MxA). This phenotype confers to this cell line a viral resistance that is able to block the oncolysis mediated by a wild type Adenovirus5 (Ad5) as well as the GFP expression Ad5 mediated (Monsurrò V, et al. *J Transl Med.* 2010;8:10).However, by further analysis, we are now showing that the parental cell line presents, by looking in immunohistochemistry the MxA distribution, two different subgroups, one positive and one negative for the selected marker.

In human prostate carcinoma cell line PC3 was demonstrated the existence of different subpopulations, that once cloned, showed a different self-renewing Tumor-Initiating ability (Hangwen L, et al. *Cancer Res.* 2008;68).In order to analyse whether there is an association of the ISG phenotype and the viral resistance and in order to assess whether ISGs phenotype is constantly maintained or is regenerated or lost during the tumor expansion, we subcloned the HPAFI pancreatic cancer cell line and isolate and analysed the different subtypes of cells.

We obtained 13% of meroclones, 43% holoclones and 43% paraclones. The holoclones kept a positive phenotype for MxA during the in vitro expansion. The paraclones showed lower MxA positivity during the early passages, however the MxA expression increased during the in vitro culture. The Meroclones looked different according to the staining for MxA. In fact, among the 3 clones that we selected for further analysis, two were positive for MxA, while the third one lost the MxA marker in the late passages.Interestingly all the meroclones obtained survived till the late passages while 50% of holoclones and paraclones aborted during the early passages.

We analysed the ablity of the subclones to resist to viral Ad5 infection and GFP production. We found that only in one out of 7 clones tested the resistance seems to be reverted, in fact, the holoclone III12D is susceptible to the infection based on GFP expression analysis.

In conclusion we were able to subclone different populations of human pancreatic cancer cells starting from the parental cell line HPAFI and we were able to segregate the resistance phenotype that seems to be characteristic only of a percentage of the cells composing the parental cell line.

Evaluation of HER2/NEU and Folate Binding Protein (FBP) Peptides in Immunotherapy for Endometrial Cancer

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TABLE 1.

	E75		E39	
	×1 Stim	×2 Stim	×1 Stim	×2 Stim
IFN-γ				
KLE	170 ± 55	165 ± 80	204 ± 58	126 ± 100
MEF	224 ± 82	153 ± 99	348 ± 87	235 ± 125
Granzyme				
KLE	338 ± 228	220 ± 92	367 ± 181	146 ± 90
MEF	447 ± 250	142 ± 74	502 ± 218	202 ± 100

All results are spots/1 × 10^6 PBMCs.
IFN indicates interferon; PBMC, peripheral blood mononuclear cell.

Introduction: We conducted a pre-clinical study to assess the ability of two HLA-A2 (A2) restricted peptides, E75 (Her2: aa369-377) and E39 (FBP: aa191-199), to generate anti-tumor immune responses in endometrial cancer (EnCa) patients. We measured ex-vivo peptide-induced cytokine secretion and cytotoxic activity in peripheral blood mononuclear cells (PBMC) from EnCa patients. In addition, PMBC stimulated with peptide-loaded dendritic cells (DC) were tested for their ability to elicit inflammatory and lytic activity against EnCa cell lines expressing Her2/neu and FBP.
Methods: PBMC samples obtained from 16 EnCa patients were tested for A2 expression. A2 positive samples were analyzed for peptide-specific stimulation of IFN-γ and granzyme secretion ex vivo via the ELISPOT assay with subtracted negative controls. DC (monocyte-derived using GM-CSF and IL4) pulsed with E75 or E39 were used to stimulate PBMC cultures that were not (1 × stim) or were (2 × stim) previously stimulated with E75 or E39 peptides. These cultures were studied for IFN-γ and granzyme secretion against HLA-A2+ EnCa cell lines (KLE and MEF280) using the ELISPOT assay with subtracted negative controls. Results are reported at spots/1 × 106 PBMCs.
Results: Twelve of the sixteen (75%) EnCa patients were found to be A2 positive. In ex-vivo ELISPOT assay the mean specific response against E75 was 95 ± 42 spots for IFN and 43 ± 22 spots for granzyme and against E39 was 28 ± 17 spots for IFN and 2 ± 2 spots for granzyme. PBMC cultures stimulated once or twice with peptide-pulsed DC showed peptide-specific cytotoxic activity against HLA-A2+ EnCa cells by IFN-γ and granzyme (Table 1).
Conclusion: The peptides E75 and E39 are capable of inducing anti-tumor specific immune responses in the PBMC of EnCa patients. These peptides are being evaluated for use in vaccines in Phase I clinical trials for endometrial cancer.

Artificial Neural Network (ANN) and Immune Monitoring Analysis for Patients with Progressive Castration-Resistant Prostate Cancer Undergoing Therapeutic Tumor Cell Vaccination

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Background: Prostate cancer has been historically regarded as a non-immunogenic cancer but several clinical trials using different vaccine strategies have induced anti-tumor immunity and moderate response rates. Here, a clinical phase I trial was established to determine the safety and efficacy of the allogeneic, gene-modified LNCaP cell line, expressing recombinant IL-2 and IFN-γ.
Methods: In a dose-escalating study patients (n = 30) were scheduled to receive four intradermal injections bi-weekly and a boost at day 92, and in the absence of disease progression every 3 months. Primary objectives were safety and toxicity, the determination of the PSA doubling-time (PSA-DT), and secondary objective was the immune monitoring together with ANN analysis.
Results: No dose-limiting or autoimmune toxicity was seen and during vaccination there was a significant prolongation of the PSA-DT (63 to 114 d, $P < 0.01$; intention-to-treat). Several patients (12/30) showed a PSA stabilization of at least 12 weeks together with stable bone scans, and three patients sustained a > 50% decrease in PSA versus baseline. By ELISPOT analysis an increase in antigen-specific T cells of at least 2-fold was observed. As no correlation could be drawn by simple comparison between PSA course and immune monitoring data, ANN analysis in addition to conventional statistical methods was performed. Patients were devided into two groups, with group 1 (n = 12) showing a PSA plateau for at least 12 weeks together with stable metastatic state and group 2 (n = 18) showing a shorter or no PSA plateau. For these two groups a significant difference in their reactivity against survivin ($P = 0.007$) was observed after the third vaccination. In all patients the frequency of natural regulatory T cells (Tregs) was the same as in normal healthy persons.
Conclusion: In summary, our vaccination approach with an allogeneic tumor vaccine can prolong the PSA-DT. The impact of this type of vaccination on the survival of the patients must be further studied.

Frequency of Circulating Regulatory T Cells and Tumor-Specific Effector T Cells in Patients with Metastatic Renal Cell Carcinoma Undergoing Therapeutic Tumor Cell Vaccination

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Background: New classes of angiogenesis inhibitors were applied for patients with metastatic renal cell carcinoma (mRCC). Some tumors show regression in imaging, but most patients develop resistance over time. Therefore, there is still an important need for therapeutic generic vaccines that could be applied to many patients. For vaccine development we used a well-characterized allogeneic gene-modified RCC-26 tumor cell line that showed strong immunogenic potential in extensive preclinical studies through expression of CD80 and IL-2.
Methods: The vaccine RCC-26/CD80/IL-2 was tested in a clinical phase I trial to evaluate safety and feasibility with stage IV mRCC patients matched for HLA-A*0201 allotype. Fifteen patients were enrolled in this study and the vaccine was applied s.c./i.d. in increasing doses of up to 40 mio. cells over 22 weeks.
Results: 50% of the progressing patients showed a stable disease for time periods ranging from 23 to 187 weeks. Median time to

progression was 5.3 months and median tumor-specific survival time was 15.6 months. Interestingly, studies of much larger groups of patients showed same PFS times with sorafenib or IFN-alpha, however with side-effects that are much greater. Immune monitoring revealed that vaccine-induced T cell responses could be detected in a majority of the patients with high responses to vaccine lysates which increased in 9 patients during vaccination. The majority of patients acquired increased effector T cell reactivity to several surrogate peptides that were derived from shared tumor-associated antigens. Surprisingly, a statistical significant decrease of natural Tregs was also observed in these patients during vaccination.
Conclusion: The feasibility of this vaccine study at low cost, saving time and logistics-alongside low toxicity and immune-stimulatory potential supports further evaluation in combination therapies and as adjuvant therapy in patients with minimal residual disease.

Antibody Response Following Immunotherapy Identifies Novel Antigens that are Expressed by Circulating Tumor Cells in Men with Hormone-Refractory Prostate Cancer

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A major obstacle to monitoring the immune response to immunotherapy is the wide spectrum of potential targets the immune response may recognize. Even vaccination with peptide may lead to epitope spreading against an unrelated or unknown tumor antigen. Given these possibilities, how is it possible to study the immune response? We hypothesize that development of a strong T cell response will lead to the generation of a B cell response against the same antigen. Therefore, identification of a new antibody response following immunotherapy may provide a surrogate for generation of an anti-tumor T cell response. To begin to address this hypothesis a protein array (8217 human proteins spotted in duplicate, Invitrogen) was used to assess the spectrum of antibodies generated by immunotherapy. The Phase I/II clinical trial randomized patients to GVAX immunotherapy (two prostate cancer cell lines that secrete GM-CSF, Cell Genesys Inc.) alone or in combination with nonmyeloablative chemotherapy and adoptive transfer of PBMC. Pre and week 11 aphereses were obtained to elutriate monocytes and cryopreserve PBMC for immune monitoring. Comparing pre and week 11 sera generated a top 50 "hit" list of proteins recognized by a strong post-treatment antibody response in 2 or more patients. Next we sought to determine whether these genes were expressed by the patient's tumor. Since no tumor biopsies were available, a method was developed to enumerate circulating tumor cells (CTC) in cryopreserved blood samples (Cell Search, Vedidex). We subsequently FACS sorted CTC, isolated and amplified RNA and performed RT-PCR for three genes, not previously described as tumor-associated antigens, that were identified by a strong post-treatment antibody response. Expression of these three genes was confirmed in pretreatment CTC, documenting that the immune response was targeting a potentially relevant target. Current experiments are evaluating whether a T cell response developed against the specified antigens. In conclusion, this strategy combines protein array and CTC analysis to provide an innovative approach to identify an immune response against previously undescribed targets. We predict this approach will help explain the mechanism of action of successful immunotherapy. Supported by The Prostate Cancer Foundation, DAMD PC02009, Robert W. Franz and the Chiles Foundation.

Immunological Findings in a Phase II Immunotherapy Study Using Allogeneic Lung Cancer Cells Modified to Express Alpha(1,3)Gal Epitopes in Patients with Advanced Non-Small Cell Lung Cancer

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Alpha(1,3)-galactose (aGal) epitopes are highly immunogenic in man. In animal models, vaccination with aGal expressing allogeneic tumor cells induced tumor rejection and improved survival. In a phase I/II trial immunotherapy study, we evaluated the safety and immunogenicity of vaccination with allogeneic human lung cancer cells genetically modified to express aGal epitopes (HyperAcute®-HAL) vaccine. The immunological monitoring consisted of testing for anti-aGal Ab and anti-CEA Ab by ELISA. HAL vaccines express cell surface CEA. The cellular immunological response was evaluated by ELISPOT (IL-5 and IFN-γ). Serum samples were collected before and after immunization at several time points. PBMC were collected before immunization, after patients receive at least 4 and 8 vaccines. PBMC were co-cultured with autologous DC pulsed with apoptotic/necrotic tumor cells from 4 NSCLC cell lines, three of which were the parental cell lines used to generate HAL vaccine. The fourth cell line that was not part of the HAL vaccine. As expected all patients had anti-aGal Ab before immunization.
After immunization all patients responded increasing the anti-aGal Ab 2-to 100 folds. Preliminary analysis suggests that maintaining the anti-aGal Ab levels after immunization might be a correlative biological marker with survival. On the other hand data suggests that the fold increased in the anti-aGAL Ab had no apparent correlation with better survival. Thirty one patients were tested for the presence of anti-CEA Ab and 19 patients responded with significant increased anti-CEA Ab values after immunization. Analysis of the overall survival of patients analyzed suggests no correlation in patients responding increasing the anti-CEA Ab compared to patients with no change in the anti-CEA Ab levels. We detected vaccine induced IFN-γ production after immunization in 13 patients out of 18 patients evaluated. Data indicates that IFN-γ response correlates with favorable overall survival. The majority of the patients tested have detectable IL-5 reactivity induced after vaccination. Patients responding with higher levels of IFN-γ and IL-5 also showed reactivity to H522, a lung cancer cell line non-component of HAL, suggesting that reactivity to shared tumor antigens might be induced after vaccination with Hyper-Acute vaccines to patient's own tumor cells. In conclusion data suggest that cytokines induction after vaccination and the persistence but not the fold increased in the anti-aGal values correlated with better survival in tested patients.

Exosome-targeting of Tumor Antigens Expressed by MVA-BN®-PRO Improves Antigen Immunogenicity and Enhances Anti-Tumor Efficacy

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Exosomes are small, 50 to 100 nm diameter vesicles secreted by most cell types. They have become the subject of increasing interest due to studies demonstrating activating effects of exosomes on immune cells through several different mechanisms. Furthermore, the immunogenicity of antigens can be improved when targeted to localize to exosomes.[1] This targeting can be achieved by engineering in-frame fusions of antigens with the C1C2 domain of a protein naturally localized to exosomes called Lactadherin.
MVA-BN®-PRO is a candidate immunotherapy product for the treatment of prostate cancer that has shown biological activity in Phase I clinical trials (McLeod et al. Abstract iSBTc 2010). MVA-BN®-PRO encodes two transgenic antigens, prostatic acid

phosphatase (PAP) and prostate-specific antigen (PSA), and is derived from a clonal isolate of the highly attenuated Modified Vaccinia Ankara virus stock known as MVA-BN®. To test if exosome-targeting would improve the immunogenicity of PAP and PSA, two additional versions of the vector were produced, targeting either PAP (MVA-BN®-PAP-C1C2) or PSA (MVA-BN®-PSA-C1C2) to exosomes, while leaving the second transgene untargeted. Treatment of mice with MVA-BN®-PAP-C1C2 led to a striking increase in the immune response against PAP. Anti-PAP antibody titers developed more rapidly and reached levels that were 10 to 100-fold higher than mice treated with MVA-BN®-PRO. Furthermore, treatment with MVA-BN®-PAP-C1C2 increased the frequency of anti-PAP T cells 5-fold compared to treatment with MVA-BN®-PRO. These improvements translated into a greater frequency of tumor rejection in a PAP-expressing solid tumor model. Likewise, treatment with MVA-BN®-PSA-C1C2 increased the antigenicity of PSA as compared to treatment with MVA-BN®-PRO, but to a lesser extent than that of targeting PAP to exosomes. Treatment with MVA-BN®-PSA-C1C2 resulted in significant anti-tumor efficacy in a PSA-expressing tumor model, with a trend of improved efficacy compared to MVA-BN®-PRO. These experiments confirm that targeting antigen localization to exosomes is a viable approach for improving the therapeutic potential of MVA-BN®-PRO in humans.

Reference

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In Situ CTLA-4 Blockade Mediates Regression of Local and Distant Tumors with Minimal Side Effects

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Preclinical and clinical studies describe powerful anti-tumor effects of systemic anti-CTLA-4 (aCTLA-4) antibody therapy but also indicate that blocking immune regulatory checkpoints may precipitate autoimmunity. Herein, we compared systemic high-dose and peritumoral low-dose injection of aCTLA-4 in murine models of pancreatic (Panc02) and bladder (MB49) cancer. Tumor growth and autoimmune events were monitored. Peritumoral therapy reduced tumor growth in an inverse dose-response manner, as $3 \times 30\,\mu g$ was better than $3 \times 90\,\mu g$. Local treatment was compared with systemic administration of $200\,\mu g$ antibody and similar effects were seen. Systemically treated mice had Treg accumulation in tumor-draining lymph nodes. Lymphocytes from treated mice (irrespectively of treatment route) secreted IFN-γ upon tumor cell stimulation. Spleen-derived mesothelin-stimulated CTLs secreted IFN-γ and were significantly elevated in the systemic compared to control group. Remarkably, local therapy was effective on both distant Panc02 tumors as well as on an irrelevant MB49 tumor, demonstrating its potential in metastatic disease. Systemic injection of aCTLA-4 was associated with elevated autoantibody levels, increased incidence of splenomegaly and acute gastritis compared with local treatment. Circulating CTLA-4 antibody levels were measured in serum and correlated with side effects. Taken together, in situ immunomodulation of tumors with CTLA-4 blocking antibody can mediate regression and cure of primary as well as distant tumors at reduced toxicity.

Gene Expression Profiling Useful for Predicting Clinical Responses to Personalized Peptide Vaccination

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Introduction: The field of cancer vaccines has dramatically moved forward. However, the identification of biomarkers for selecting patients adequate for cancer vaccination would be pivotal for further development of this field. To identify reliable biomarkers, we characterized gene expression profiles (transcriptome) in patients with advanced prostate cancer undergoing the personalized peptide vaccination.

Methods: Gene expression profiles were characterized by the DNA microarray technology (Illumina; total 30,000 probes) in PBMC from patients that showed better or worse responses to the peptide vaccination (survival time > 900 or < 300 d, respectively; $n = 20$ each). Statistical analysis was performed to select genes that help predict patient clinical responses. In addition, to validate the result, 10 additional PBMC samples were analyzed in a blind test.

Results: Detailed statistical analysis demonstrated that the gene expression profiles were clearly different between responders and non-responders to the peptide vaccination. A combination of 12 genes was selected as the predictive biomarker that are significantly correlated with patient survival. In the blind test, the identified genes discriminated responders from non-responders to the peptide vaccination at high accuracy.

Conclusion: The identified gene profiles may be useful as a biomarker in selecting patients adequate for the personalized peptide vaccination. Detailed characterization of individual genes identified is currently underway. The gene expression profiling in PBMC would be a breakthrough in cancer vaccine development.

Targeting HGF Impacts Both Melanoma Cells and Lymphocytes: Implications for Clinical use of HGF Blockade

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Introduction: Hepatocyte growth factor (HGF) binds its receptor c-Met and stimulates proliferation, migration, and angiogenesis. It has been implicated in oncogenesis and tumor progression in several cancer types, including melanoma. HGF also has effects on immune cells, particularly induction of monocyte migration and blockade of dendritic cell antigen presentation. Its effects on lymphocytes are less well established. While melanoma cells, monocytes and dendritic cells express c-Met, T cells do not. Therefore, we hypothesized that blockade of HGF would impair melanoma cell proliferation without affecting lymphocyte function.

Methods: Melanoma cells and PBMCs were analyzed for c-Met expression by immunoblotting. To test dependence on HGF for cell proliferation, melanoma cells and lymphocytes were treated separately with HGF antibody for 48 hours. Both cell types were analyzed after 6 and 18 hours of HGFab treatment for expression of total and phosphorylated c-Met and downstream proteins MAPK, AKT, mTOR, FAK, STAT3, STAT1, and Src.

Results: Activated c-Met was detected in melanoma cells and PBMC. At 6 hours, HGF antibody treatment decreased phosphorylation of c-Met, STAT3, STAT1, and Src in melanoma cells. By 18 hours, expression of phosphorylated AKT, MAPK, mTOR and FAK were also suppressed. In PBMC, HGFab decreased phosphorylation of c-Met by 6 hours but did not impact phosphorylation of MAPK, AKT, mTOR, STAT3, STAT1 or Src. FAK and phospho-FAK were not detected in PBMC.

Treatment of melanoma cells with HGFab inhibited proliferation of all melanoma cell lines compared to isotype control antibody, and caused net cell death for 5 of 6 melanoma cell lines. Treatment of CD3/CD28-stimulated PBMC with HGFab inhibited proliferation of two normal donor PBMC samples as compared to isotype control antibody.

Conclusions: Melanoma cells depend on autocrine signaling through c-Met, which is mediated through MAPK, STAT3, AKT and mTOR. It isn't surprising that c-Met mediates downstream activation of these proteins, but the degree of inhibition suggests potential importance of HGF in the malignant melanoma phenotype. Consistent with these signaling changes, melanoma cell proliferation also is inhibited by HGFab. In PBMC, HGFab

did not change phosphorylation of MAPK, AKT, STAT3, STAT1 or mTOR. Importantly, HGFab inhibited proliferation of PBMC to a similar degree as melanoma cells. These data suggest that HGFab has promise as anti-melanoma therapy, but has potentially negative effects on immune cells. Our results indicate this may be mediated by different pathways than in melanoma cells. Differences in these signaling pathways may be exploited by targeted therapies and need to be defined before combining immune therapy and anti-HGF therapy.

Molecular Basis of Aberrant Expression of Alkaline Phosphatase in Renal Brush Border Membrane from Renal Cell Carcinoma Patients

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Background: The incidence of renal cell carcinoma (RCC) has been increasing worldwide. It continues to present a diagnostic and therapeutic challenge. It accounts for approximately 3% of adult malignancies and 90% to 95% of neoplasm arising from kidney. RCC originates from proximal tubular epithelium of adult kidney. Alkaline phosphatase is abundantly expressed on the BBM and serves as an excellent marker for its integrity. Earlier reports have demonstrated that the activities of brush border membrane (BBM) enzymes are altered in cancer cells. The present study was conducted to find out the molecular basis of altered expression/activity of ALP in BBM from RCC in comparison to normal renal BBM.

Methods: Total 30 histopathologically confirmed cases of RCC were included in the present study. The activity of alkaline phosphatase was determined by enzymatic assay. The expression of alkaline phosphatase at protein level was determined by western blot and at expression level by RT PCR study.

Results: The specific activity of ALP was drastically reduced in homogenate as well as in BBM from RCC as compared to BBM from adjacent normal kidney parenchyma.

SDS-PAGE study showed that the BBM proteins of higher molecular weights were poorly expressed in BBM from RCC as compare to BBM from adjacent normal kidney parenchyma. Incubation of gel with BCIP/NBT dye showed that the expression of ALP in BBM from RCC was reduced as compared to adjacent normal kidney BBM. Western blot analysis using anti ALP antibody also confirmed the reduced expression of ALP in RCC BBM. Further, to check whether this reduced expression of ALP occurs at mRNA level, a semi quantitative RT-PCR was done, which showed markedly reduced expression of ALP mRNA in RCC as compared to adjacent normal kidney.

Conclusion: The reduced expression/activity of alkaline phosphatase in renal brush border membrane was due to reduced expression of alkaline phosphatase at transcriptional level.

Overcoming BRAF Inhibitor Resistance in Melanoma

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The discovery that ~50% of human melanomas harbor activating V600E mutations in the serine/threonine kinase BRAF has raised the possibility that these tumors may be amenable to targeted therapy. The importance of mutated BRAF for the growth and survival of melanoma cells has since been validated in a large number of pre-clinical studies and clinical trials of the novel BRAF kinase inhibitor PLX4032 are now underway. Initial results from the phase II extension trial of PLX4032 in melanoma patients selected for the BRAF V600E mutation are highly encouraging, with responses reported in an unprecedented 81% of those treated. Although the clinical development of BRAF inhibitors is at an early stage, it is already clear that the impressive levels of response seen initially do not necessarily persist for extended periods of time. Recent data from our group and others suggests that melanoma cells re-wire their intracellular signaling when BRAF is inhibited and use parallel signal transduction pathways for their growth and progression. This presentation will discuss some of the putative mechanisms by which BRAF-mutated melanoma cells escape from BRAF inhibitor therapy and will outline how these contribute to both intrinsic and acquired resistance. It is expected that an enhanced understanding of the signaling cross-talk mechanisms present in melanoma cells will allow combination therapy strategies to be designed which will either delay or abrogate the onset of resistance when BRAF is inhibited.

Superior Killing Characteristics of a Tumor-Targeted, Genetically Encoded Trail Trimer (TR3)

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Background: Cancer cells often develop resistance to one or more apoptotic pathways. Recently it has been shown that even within a population of homogenous cancer cells, stochastic processes render some cells refractory to monotherapy. As a result, it is believed that effective cancer therapeutics cannot be monotherapies but must be combined to overcome both evolved and stochastic resistance. TRAIL is an endogenous TNF-superfamily member that causes apoptosis via the extrinsic death receptor pathway. Unfortunately, TRAIL-mediated cell death is frequently incomplete even in the presence of an increased cell surface density of these receptors. Fortunately, cancer cells often express other, secondary tumor antigens on their surface, which are absent or much reduced on normal host cells and could be utilized to deliver TRAIL more specifically to the tumor microenvironment.

Methods: We recently developed a novel TRAIL form, designated TR3, which represents a fusion protein of three consecutive TRAIL ectodomains that is generically extensible with stoichiometric control. Besides its improved stability over non-covalently associated TRAIL trimers, we provide here an example of adding a single chain antibody (scFv) to the N-terminus of TR3 that specifically recognizes the tumor marker mesothelin, an antigen that is highly expressed in a number of human malignancies including pancreatic cancer. We generated two mesothelin targeted TR3 fusion proteins: one spacer containing (scFv-S-TR3) and one spacer-deficient (scFv-TR3) and assessed apoptosis induction following transient expression of mesothelin in cancer cell lines.

Results: Non-targeted TR3 showed no bias toward mesothelin-expressing target cells. In contrast, scFv-S-TR3 killed ~25% more mesothelin-positive cells compared to non-transfected controls. As predicted, the spacer played a pivotal role in mediating the improved killing capacity of scFv-S-TR3.

Conclusions: We have generated a cancer-specific TRAIL form that associates with surface-expressed mesothelin and, at the same time, facilitates engagement with surface-exposed death receptors. The spacer, introduced between the scFv and TR3 was absolutely required for this augmented killing effect. The ability to simultaneously bind a cancer drug selectively to a tumor antigen and induce stronger (or more sustained) signaling events that lead to superior target cell death raises the possibility of using tumor-targeted TR3 as a monotherapy.This work was supported by NIH grants 5P30CA9184208 and 1R21CA150945.

Spontaneous Cytotoxic T cell Reactivity Against IDO Exists in Cancer Patients and in Healthy Donors

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Indoleamine 2,3-dioxygenase (IDO) is an immunoregulatory enzyme that is implicated in suppressing T cell immunity in normal and pathological settings, including cancer. IDO regulates immune

responses through the capacity to degrade the essential amino-acid tryptophan into kynurenine and other downstream metabolites that suppress effector T cell function and favour the differentiation of regulatory T cells. IDO can be expressed by a variety of cell types, including dendritic cells (DC), tumor cells and stromal cells. IDO is widely dysregulated in tumors and tumor-draining lymph nodes, where it can mediate immune tolerance to tumor antigens and facilitate immune escape. Recently, we showed that IDO itself is subject to cellular immune responses. Hence, spontaneous cytotoxic T cell reactivity against IDO is present in peripheral blood as well as in the tumor microenvironment of different cancer patients. We demonstrated that these IDO reactive T cells are indeed peptide specific, cytotoxic effector cells. Hence, IDO reactive T cells are able to recognize and kill tumor cells including directly isolated AML blasts as well as IDO-expressing dendritic cells, that is, one of the major immune suppressive cell populations. Consequently, IDO may serve as an important and widely applicable target for anti-cancer immunotherapeutic strategies. Here, we describe that spontaneous cytotoxic T cell reactivity against IDO also exists in healthy individuals. We show that such IDO-specific T cells boost immunity against viral antigens by eliminating IDO+ suppressive cells. This has profound effects on the balance between IL-17-producing CD4+ T cells and regulatory T cells. Furthermore, IDO-specific T cells can be induced in healthy individuals by non-specific inflammatory stimuli such as IFN-γ and IL-2. In the clinical setting, IDO may serve as an important and widely applicable target for immunotherapeutic strategies where IDO constitute a significant counter-regulatory mechanism induced by pro-inflammatory signals. Regulatory T cells have so far been defined as a specialized subpopulation of T cells that act as suppressor T cells. However, here we describe effector T cells with a general regulatory function that may play a vital role for the mounting or keeping of an effective adaptive immune response.

Pentostatin Plus Cyclophosphamide to Eliminate Immunogenicity of Anti-Mesothelin Immunotoxin (SS1P)

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The immunotoxin (IT) SS1P is composed of an anti-mesothelin Fv fused to a 38 kDa fragment of Pseudomonas exotoxin A and is being evaluated in mesothelioma and lung cancer. But in mesothelioma patients, neutralizing antibodies develop after 1 cycle of therapy, thereby preventing additional treatment cycles. IT therapy has been shown to be effective in hairy cell leukemia, where multiple cycles can be given without neutralizing antibody formation. This lack of immunogenicity may result from immune dysfunction, either from the disorder itself or from previous immune depleting therapy. In this study, our aim was to determine if using chemotherapy to dysregulate the immune system, specifically comparing immune depletion to immune suppression, might abrogate immunogenicity. BALB/c mice were injected with 10 µg SS1P weekly for 3 weeks and serum was evaluated for neutralizing antibodies at the end of each week. An immune depletion group was treated for six days prior to the first IT immunization with daily cyclophosphamide (50 mg/kg/d) and pentostatin (1 mg/kg/d) every other day, followed by weekly maintenance doses of P/C one day prior to immunization. An immune suppression group was treated on an every other day schedule of rapamycin (3 mg/kg/d) for the entire post-immunization period. Three weeks after the first immunization spleen cells were isolated, counted and analyzed by flow cytometry to determine the levels of depletion of CD4+ and CD8+ T cells, B cells (B220+), and myeloid cells (Gr-1+). Immunogenicity was determined by presence of antibodies to SS1P by ELISA (DC) or conformational ELISA (ICC) assays. Both the regimens of P/C and R were safe and well tolerated in the mice in terms of weight loss and survival. The P/C regimen markedly depleted both T and B cells with relative sparing of myeloid cells. Readouts of immunogenicity by the ICC assay showed 5/10 mice developed antibodies in the IT only group, while no mice (0/10) developed antibodies in either the P/C and R treated groups. However, the DC assay revealed 3/10 mice treated with R indeed had anti-SS1P antibodies present but 0/10 P/C-treated mice showed immunogenicity. Although immune suppression by treatment with rapamycin did cause reduced immunogenicity, antibodies to the anti-mesothelin IT were detectable at low levels. On the other hand, immune depletion with pentostatin plus cyclophosphamide prevented formation of any detectable IT-targeted antibodies. These findings will allow for the investigation of immune depletion with these drugs as a mechanism to overcome immunogenicity in treatment of mesothelioma with the immunotoxin SSIP. Since mesothelin is also expressed on lung, ovarian and pancreatic cancer, results could be applicable to treatment of these more common cancers as well.

Humanization of a High Affinity, Neutralizing Antibody Against Hepatitis B Surface Antigen

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Hepatitis B virus chronically infect more than 500 million people worldwide and account for about two-thirds of all hepatocellular carcinoma, the third most common cause of cancer-related death. Universal vaccination using Hepatitis B surface antigen (HBsAg) is a possible way to achieve eradication of the disease. However, alternative strategies to combat hepatitis B are needed because of the existence of vaccine non-responders, emergence of viral escape mutants and the persistence of a subpopulation of viral carriers in developing countries. We previously generated a potentially neutralizing mouse monoclonal antibody (5S) against HBsAg. This antibody showed very high affinity and stable binding in presence of different agents that usually destabilize antigen-antibody interactions. In this report we describe the humanization of 5S antibody by grafting its antigen binding site onto framework of the human consensus sequence of highest similarity. We have used molecular modeling to alter not only the clearly permissible residues but also several minimal positional template and VH/VL interface residues. The humanized antibody retained a high binding affinity (KD = 1.27 nmol/L) to HBsAg and bound to the same epitope of HBsAg as the parent molecule. This high affinity humanized antibody provides a basis for the development of therapeutic molecules that can be safely utilized for the prophylaxis and treatment for Hepatitis B infection.

Development of Cancer Stem Cell Vaccine

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Cancer stem cells (CSCs), a small population of cancer cells with stem cell-like phenotype and tumor-initiating capacity, are important targets for cancer therapy because they may be responsible for recurrence after chemotherapy. We are exploring the possibility for development of cancer vaccine targeting CSCs. In order to clarify immunopathological properties of CSCs, CSCs with high tumor-initiating capacity were collected from various cancer cell lines by sorting a side population (SP), which has a capacity to pump out Hoechst 33342 dye through ABC transporters. By comparative gene expression analysis of SP and main population (MP) cells derived from lung, breast and colon cancer cell lines, several CSC genes were identified, including SOX2, SMCP, DNAJB8 and OR7C1. These genes were expressed preferentially in SP cells, but barely detected in normal adult tissues except for testis. Overexpression of the genes increased tumor-initiating capacity of cancer cells in vivo, whereas RNAi-mediated

knockdown of the genes dramatically decreased the tumorigenic capacity, indicating that the genes could be associated with tumor-initiating capacity of CSCs. We could induce cytotoxic T cells (CTLs) from peripheral blood lymphocytes of cancer patients after in vitro stimulation with SOX2-derived peptides. SP cells were susceptible to cytotoxicity of CTLs, whereas they were resistant to chemotherapeutic drugs. In addition, DNA vaccine encoding the CSC gene showed higher tumor suppressive capacity in vivo in a mouse tumor model. Our studies indicate that cancer vaccine targeting CSCs might serve as potent immunotherapy for cancer.

Evaluation of FOXP3 Microsatellite Polymorphisms in High-Risk Melanoma Patients Receiving Adjuvant Interferon

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Background: Attempts to identify patients who benefit from adjuvant treatment with interferon alfa-2b (IFN) have been disappointing. The development of autoimmunity during adjuvant therapy with IFN cannot assist selection of patients for therapy at baseline. The FOXP3 is located at Xp11.23 within an area of autoimmune disease linkage and therefore is an excellent positional candidate gene for autoimmunity at this locus. Consequently we performed a microsatellite analysis of FOXP3 gene in the exon 0 region in high-risk melanoma patients enrolled in a study of two regimens of high-dose IFN.

Methods: A fragment analysis was performed in the DNA of 259 stage IIb, IIc and III melanoma patients. After amplification and electrophoresis run, data from each sample were analyzed using Genemapper software (Applied Biosystems) which objectively calls the fragments size.

Results: Thirteen alleles were detected (size: 284, 286, 288, 290, 292, 298, 302, 304, 306, 310, 312, 314, 316). The tabular distribution of these alleles in melanoma patients is presented below (Table 1). At a median follow up of 71 months (range 7.1 to 138.7 mo), 144 patients have recurred and 95 have died. There were no statistically significant differences in the incidence of FOXP3 alleles between melanoma patients who recurred and those with no evidence of recurrence. RFS did not differ significantly between patients with recurrence and those without for each of the alleles detected.

Conclusions: No allele defined by the microsatellite analysis of the FOXP3 gene in exon 0 was correlated with improved RFS in this high-risk group of melanoma patients.

TABLE 1.

Genes	No Relapse, N = 115 (%)	Relapse, N = 144 (%)	*P*
Allele_284	2 (1.74)	2 (1.39)	1
Allele_286	2 (1.74)	1 (0.69)	0.586
Allele_288	79 (68.70)	90 (62.50)	0.358
Allele_290	4 (3.48)	2 (1.39)	0.411
Allele_292	0 (0.00)	1 (0.69)	1
Allele_298	1 (0.87)	5 (3.47)	0.231
Allele_302	1 (0.87)	3 (2.08)	0.632
Allele_304	1 (0.87)	2 (1.39)	1
Allele_306	6 (5.22)	11 (7.64)	0.463
Allele_310	16 (13.91)	18 (12.50)	0.853
Allele_312	1 (0.87)	5 (3.47)	0.231
Allele_314	2 (1.74)	2 (1.39)	1
Allele_316	0 (0.00)	2 (1.39)	0.504

Reshaping CD4 and CD8 Memory T Cell Proliferation by Treating Cancer Patients with an OX40 Agonist: Immunologic Assessment of a Phase I Clinical Trial

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OX40, a member of the TNF superfamily, is a potent co-stimulatory molecule expressed upon activation at the surface of CD4 and CD8 T cells. Its engagement improves T cell effector function and survival. Preclinical studies have shown that OX40 agonists injected into tumor-bearing mice increase anti-tumor immunity leading tumor-free survival in several mouse models. These results prompted the initiation of phase I clinical trial using a mouse anti-human OX40 agonist antibody in patients diagnosed with a variety of solid malignancies. In this dose-escalation study, the agonist OX40 antibody was administered on Day 1, 3 and 5 at 0.1, 0.4 and 2 mg/kg, respectively. Immune monitoring analysis, over a 2 month period, was performed on PBL by flow cytometry directly ex vivo, using a 10-color antibody panel detecting CD3, CD4, CD8, CD95, CD28, CD25, CD127, CCR7, FoxP3 and Ki-67. On Day 8-15, we have observed a 2-3-fold increase in the proliferating CD4+ CD95+ T cells as detected by increased Ki-67 staining, mostly in the FoxP3- population with no significant increase in CD4+ FoxP3+ T cells proliferation (Treg). The proportion of cycling CD8+ CD95+ T cells peaked later 15-29 days after the administration of the antibody with a 2-4.5-fold increase of cycling cells compared to a group of nine controls. Interestingly, the middle dose (0.4 mg/kg) showed the greatest sustained increase in proliferation for both the CD4 (non-Treg) and CD8 memory T cell population. Administration of anti-OX40 antibody, not only triggered T cell proliferation, but also increased the activation status of the CD8+ T cells as measured by the co-expression of CD38 and HLA-DR on the cycling cells. Using these two surface markers, cycling CD8+ T cells were sorted and a gene array analysis showed increases in mRNA for CD86, CTLA-4, and 4-1BB when compared to the non-cycling CD8+ T cells. Flow cytometry data confirmed that Ki-67+ CD8+ T cells were enriched for these markers. Finally, in 2 out of 3 patients, where autologous tumor was obtained the infusion of anti-OX40 antibody appeared to increase the proportion of tumor specific T cells. PBMC collected pre and post anti-OX40 administration were co-cultured with melanoma cell lines. CD8 T cells, from PBMC obtained after anti-OX40 administration showed an increase in INF-γ secretion toward autologous or HLA-matched melanoma cell lines. These results suggest that administration of an anti-OX40 antibody in cancer patients increases tumor-specific CD4+ and CD8+ T cells by enhancing their proliferation and the production of type I cytokines.

Characterization of Antigen Specific T Cell Activation and Cytokine Expression Induced by Sipuleucel-T

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Sipuleucel-T (PROVENGE®) is an autologous cellular immunotherapy designed to stimulate an immune response to prostate cancer. PA2024 [a recombinant human antigen consisting of prostatic acid phosphatase (PAP) and granulocyte macrophage-colony stimulating factor (GM-CSF)] is cultured ex vivo for 2 days with peripheral blood mononuclear cells (PBMCs) isolated from a standard leukapheresis procedure. Sipuleucel-T obtained at Wks 0, 2, and 4, after which cells are infused back into subjects. This study examined the T cell activation profile and cytokine production in sipuleucel-T from men with asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer enrolled in a Phase 3 study (D9902B, IMPACT). PBMCs from each leukapheresis were incubated with either PA2024 or GM-CSF (sargramostim),

and evaluated for the production of cytokines. The PA2024 culture supernatants at each treatment week displayed a substantial increase in both APC activation-associated cytokines (IL-1-alpha, IL-10, IL-12p70, IFN-gamma and TNF-alpha) and T cell activation-associated cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-gamma and TNF-alpha), which increased at the second and third infusions relative to the first. In contrast, the GM-CSF culture supernatants had comparatively diminished levels of both sets of cytokines at all treatments. In additional experiments, both CD4+ and CD8+ T cells generated by culture with either PA2024 or GM-CSF were assessed by flow cytometry for the expression of CD134 (OX40), CD137 (4-1BB), CD278 (ICOS) and CD279 (PD-1). Cells from the culture with PA2024, but not with GM-CSF, displayed a generalized pattern of enhanced expression of the T cell activation markers at the second infusion compared to the first and third.
These data indicate that T cell activation and enhanced cytokine expression are a consequence of priming after the first infusion. These effects are not driven by GM-CSF, as T cell activation and enhanced cytokine production were only observed in PA2024 cultures.

Impact of HLA-A2 Status on Ipilimumab Efficacy and Safety: Pooled Data from Four Phase II Trials in Advanced Melanoma

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Ipilimumab, which blocks cytotoxic T-lymphocyte antigen-4 to potentiate an antitumor T cell response, has demonstrated an improvement in overall survival (OS) in a phase III, randomized controlled trial in previously treated patients with advanced melanoma.[1] In this trial, ipilimumab at 3 mg/kg, with or without an HLA-A2-restricted gp100 peptide vaccine, was compared to gp100 alone. The mechanism of action of this gp100 vaccine required that all patients be HLA-A2+. However, the mechanism of action of ipilimumab suggests that HLA status has no impact on its clinical activity. We analyzed data from phase II trials to determine if HLA-A2 status impacts ipilimumab efficacy and safety.
Data were pooled from 4 completed phase II trials (CA184-004, -007, -008, and -022) for previously treated patients with advanced melanoma who received ipilimumab at 0.3, 3 or 10 mg/kg, and were analyzed comparing HLA-A2+ and HLA-A2− patients. In phase II studies, HLA-A2 typing was performed for biomarker analyses but was not used to restrict eligibility. Using a PCR-based assay, on-study HLA-A2 typing was performed on 453 previously treated patients: 187 (41.3%) were HLA-A2+ and 266 (58.7%) were HLA-A2-. A similar median OS was observed for HLA-A2+ and HLA-A2- patients treated with ipilimumab across studies (Table 1).[1]
The adverse events associated with ipilimumab treatment are primarily immune-related, which reflect its immune-based mechanism of action.[1] Ipilimumab-associated irAEs can be severe and life-threatening, but most are reversible using product-specific treatment guidelines.[1] Safety analyses on data from all treated patients in the phase II trials and in patients treated with ipilimumab alone in the phase III trial showed that irAEs occur at similar frequencies in HLA-A2+ and HLA-A2− patients (Table 1).
These retrospective analyses show that HLA-A2− and HLA-A2+ patients with advanced melanoma have a similar survival benefit with a similar toxicity profile, from ipilimumab therapy. The data suggest that HLA-A2 subtype does not impact survival outcomes with ipilimumab in advanced melanoma patients.

Reference:

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NY-ESO-1-Specific CD8 T Cell Response in NY-ESO-1 Seropositive Metastatic Melanoma Patients Treated with Ipilimumab Correlates with Clinical Benefit

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Background: Ipilimumab, a monoclonal antibody against cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), has been shown to elicit durable immunologic and clinical responses in patients with metastatic melanoma. Our lab has demonstrated that ipilimumab enhances B and T cell immunity to NY-ESO-1, a prototypical cancer-testes antigen expressed in melanoma.
Methods: In order to better characterize the association between immune response and clinical outcome, sera from 100 advanced melanoma patients treated with ipilimumab at Memorial Sloan-Kettering Cancer Center were analyzed for NY-ESO-1 seropositivity. In addition, pre- and/or post-therapy peripheral blood mononuclear cells from all NY-ESO-1 seropositive patients without the purification of CD4+ and CD8+ T cells were assayed for NY-ESO-1-specific CD4+ and CD8+ T cell responses by intracellular cytokine staining following a 10-day in vitro stimulation with NY-ESO-1 overlapping peptides.
Results: 20 out of the 100 patients were found to be seropositive at any time-point (6 seroconverted), with a trend toward these patients experiencing more frequent clinical benefit (11/20; 55%) than seronegative patients (25/80; 31%), $P = 0.067$. Within the seropositive subgroup with the availability of suitable specimens,

TABLE 1.

	Phase III	Pooled Phase II Data	
Survival	HLA-A2$^+$ (N = 137)	HLA-A2$^+$ (N = 187)	HLA-A2$^-$ (N = 266)
Median OS, months (95% CI)	10.1 (8.0-13.8)	9.3 (7.4-11.5)	11.4 (9.3-15.1)
irAEs, no. (%)	HLA-A2$^+$ (N = 131)	HLA-A2$^+$ (N = 218)*	HLA-A2$^-$ (N = 311)*
Skin	57 (43.5)	97 (44.5)	153 (49.2)
Gastrointestinal	38 (29.0)	65 (29.8)	119 (38.3)
Hepatic	5 (3.8)	9 (4.1)	23 (7.4)
Other	6 (4.6)	5 (2.3)	18 (5.8)

CI indicates confidence interval; HLA, human leukocyte antigen.
*Includes previously treated and untreated patients who received ipilimumab.

17/19 (89%) and 13/19 (68%) generated an NY-ESO-1-specific CD4+ and CD8+ T cell response, respectively. NY-ESO-1 seropositive patients who generated NY-ESO-1-specific interferon-gamma (IFNγ)+ CD8+ T cells experienced significantly more frequent clinical benefit (10/13; 77%) than those who did not mount this immune response (1/7; 14%), $P = 0.017$. No association was found between an NY-ESO-1-specific IFNγ+ CD4+ T cell responses and clinical outcome ($P = 0.16$). Furthermore, all detectable CD4+ or CD8+ IFNγ+ T cell responses showed polyfunctionality for TNFα, MIP-1β and/or CD107a. Finally, Being NY-ESO-1 seropositive with a CD8+ T cell response demonstrated a significant survival advantage compared to the general population (median survival not reached vs. 8 mo, $P = 0.0158$).

Conclusion: NY-ESO-1 seropositive patients may or may not develop NY-ESO-1 specific CD8+ T cell responses. Those who develop both antibody and CD8+ T cell responses may be more likely to experience clinical benefit. Further understanding the significance of this association could have prognostic or predictive value and may support future studies in patients previously immune to NY-ESO-1 or other relevant antigens.

Immunotherapy of Adenocarcinomas with Gc Protein-Derived Macrophage Activating Factor, GcMAF

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Intratumor BCG administration eradicates local as well as metastasized tumors. Administration of BCG into noncancerous tissues, however, results in no effect on the tumors. Inflammation induced by BCG in normal tissues releases lysophospholipids that activate macrophages. Because cancerous tissues contain alkylphospholipids, BCG-induced inflammation of cancerous tissues produces alkyl-lysophospholipids and alkylglycerols that activate macrophages approximately 400 times more effective than lysophospholipids, implying that highly activated macrophages are tumoricidal. Inflammation-primed macrophage activation is the principal macrophage activation process that requires hydrolysis of serum Gc protein (known as vitamin D-binding protein) with an inducible β-galactosidase of inflammatory B cells and the Neu-1 sialidase of T cells to yield the macrophage activating factor (MAF). Thus, Gc protein is the precursor for MAF. However, the MAF precursor activity of serum Gc protein of cancer patients was lost or reduced because Gc protein is deglycosylated by serum α-N-acetylgalactosaminidase (Nagalase) secreted from cancerous cells but not from healthy cells. Thus, serum Nagalase activity is proportional to tumor burden and serves as an excellent prognostic index. Deglycosylated serum Gc protein can not be converted to MAF, leading to immunosuppression. Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generates the most potent MAF (GcMAF) that produces no side effect in humans. In vitro treatment of macrophages/monocytes with 10 pg GcMAF activates macrophages at maximal level with 200-fold increased ingestion index and 30-fold increased superoxide generating capacity in 3 hours. Both in vivo and in vitro GcMAF activated macrophages developed an enormous variation of receptors that recognize cell surface abnormality of malignant cells and become tumoricidal to a variety of cancers indiscriminately. GcMAF also has a potent mitogenic activity on myeloid progenitor cells that generate systemically 40-fold increase in the activated macrophages in 4 days. When adenocarcinoma (metastatic breast, prostate and colorectal cancers) patients were intramuscularly administered with 100 ng GcMAF/wk, their tumors were eradicated in 16 to 25 weeks. These patients were tumor free for more than six years after GcMAF therapy. Since intravenous administration of GcMAF allows rapid interaction of GcMAF with myeloid progenitor cells in bone marrow, the systemic cell counts of the activated macrophages increased to more than 200-fold in 2 days. Weekly intravenous administration of 100 ng GcMAF to adenocarcinoma patients eradicates tumors in 11 to 16 weeks.

Anti-Tumor Action and Tolerance of Oral SHP-1 Inhibitor TPI-1a4 in Mouse Models

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Cancer therapeutic efficacy via targeting negative immune regulators has been demonstrated by ipilimumab, which extends overall survival in advanced melanoma. Protein tyrosine phosphatase SHP-1 is a key negative regulator in anti-tumor immune cells and might be targeted as a novel cancer therapeutic strategy. Indeed, tyrosine phosphatase inhibitor-1a4 (TPI-1a4) was identified recently as a novel small molecule inhibitor for SHP-1 and exhibited pre-clinical anti-tumors in mice. Its translational potential has been further investigated in the current study.

TPI-1a4 as a single oral agent inhibited the growth of murine K1735 melanoma tumors in a dose-dependent manner. The growth of 4-day established K1735 tumors (s.c.) in syngeneic C3H/HeJ mice was inhibited 44% ($P < 0.05$) by TPI-1a4 at 0.4 mg/kg (5 d/wk, po) or 90% ($P < 0.01$) at 0.8 mg/kg (5 d/wk, po). Evaluation of tumor histology demonstrated a corresponding increases (2× and 4× for the two treatments) in tumor-infiltrating lymphocytes (TIL), consistent with an immune mechanism of action. Survival of mice bearing K1735 tumors was prolonged by TPI-1a4 treatment (1 mg/kg, 5 d/wk, po) from 21 days up to 42 days. TPI-1a4 had maximal tolerated dose (MTD) > 30 mg/kg and LD50 > 60 mg/kg in CD-1 mice during a 50-day period (5 d/wk, po). Moreover, oral TPI-1a4 at 3 mg/kg was tolerated by Balb/c mice for 4 months with no apparent side effects. Consistent with a low toxicity and high efficiency in activating immune cells, TPI-1a4 at 1 to 30 μg/mL showed escalating activities in inducing mouse splenic IFNγ+ cells in vitro whereas its parental compound was less effective. Novel analogs of TPI-1a4 were synthesized and evaluated, leading to the identification of a new anti-tumor agent.

These data provide further evidences designate TPI-1a4 as an attractive platform for developing novel immunotherapeutic agents and support SHP-1 as a cancer therapeutic target.

A Flow Cytometry Method to Quantitate Internalized Immunotoxins In Vivo Explains Taxol and Immunotoxin Synergy

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Cancer cells within solid tumors live in a unique microenvironment containing barriers impairing the penetration of antibodies, immunoconjugates and immunotoxins. SS1P is an immunotoxin composed of the Fv portion of a mesothelin specific antibody fused to a bacterial toxin and is now undergoing Phase II testing in mesothelioma. We describe a new approach to study the targeting process of SS1P in tumors. A flow cytometry-based method (FC method) was developed to quantify the uptake of SS1P by individual tumor cells, and a gel filtration assay was developed to study shed mesothelin (sMSLN), a barrier for SS1P therapy. The cellular uptake of SS1P in tumor cells peaked several hours after blood SS1P was cleared, reflecting the underlining intra-tumor distribution process of SS1P independent of its blood supply. With this approach, we demonstrated Taxol improved the penetration of SS1P in the tumor, associated with a reduced sMSLN barrier in tumor extracellular fluid. Our study provides a mechanistic basis for the combined use of SS1P with cytotoxic drugs and helps explain the increased antitumor activity when chemotherapy and antibody-based therapies are combined. We also showed that the FC method can quantify the tumor cell uptake of Herceptin and an immunotoxin targeting HER2/neu. This method has the advantage in quantifying drug penetration process and the ability to study drug distribution among cellular components of solid tumor. This study provided an alternative approach to study drug penetration in solid tumor.

L-PG-CpG, a Nanopoymer Formulated Immunomodulator with Enhanced Anticancer Activity

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Melanoma is the deadliest of the skin cancers due to its propensity to widely metastasize throughout the body. Conventional therapies currently have limited efficacy against metastatic melanoma. There is now strong evidence that the immune system can play a significant role in inducing long-term benefits for some patients with metastatic melanoma. One approach towards the development of a strong immune response involves activation of innate immune cells such as plasmacytoid dendritic cells (pDC) by engaging specific toll like receptors (TLRs). TLR9 is the most specific of the human TLRs expression in pDCs and B cells that respond directly to stimulation by CpG oligodexoxynucleotide. Unfortunately, systemic injection of CpG causes activation of pDC cells in major immune organs, and exhausts the pool of this important type of anti-tumor cells outside of the tumor.

Recently, we have developed a macrophage-tropic polymer technology,[1] which interact with tumor infiltrating macrophages and accumulate in tumor sites. We hypothesize that targeted delivery of CpG to melanoma in vivo through biodegradable L-PG would effectively generate protective immunity and enhance antitumor activity while reducing or even abolishing the systemic activation of pDC. In this pre-clinical study, we have applied the macrophage-tropic polymer technology to deliver CpG ODN2216 to tumor sites in a mouse model of melanoma. We synthesized poly(L-glutamic acid)-CpG conjugate (L-PG-CpG), and examined its anticancer effect as compared to non-conjugated CpG ODN2216 when administered intratumorally to B16-OVA melanoma subcutaneous transplant. We found nanopolymer conjugated CpG ODN2216 reduced tumor growth, more than non-conjugated CpG; Furthermore, nanopolymer conjugated CpG ODN2216 triggered a stronger systemic CD8 T cell responses toward tumor antigen (OVA). Ongoing studies are being focused on determining the optimal physicochemical characteristics of nanopolyer-CpG to their immunostimulatory activities anticancer effect following intratumoral injection; developing PG-CpG nanoconstructs actively targeted to melanoma cells through both receptor-mediated uptake and tyrosinase-mediated CpG release; and enhancing antitumor immunity by rational combination of PG-CpG nanoconstructs with agonists of positive costimulatory signals and inhibitors of negative immune regulatory signals.

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VACCINE COMBINATIONS

Autologous Vaccine, Active Immunotherapy of Cancer Patients According to TNF-α, IFN-γ, sIL-2R, sIL-6R Monitoring

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Autologous Vaccine, active immunotherapy AHICE, after biochemical de-masking of the tumor-cells, is the therapeutic treatment in the optional adjuvant approaches of cancer patients management.

In the present study we report the results of lymphocytes phenotyping plus TNF-α, IFN-γ, sIL-2R, sIL-6R monitoring in relation to outcomes of AHICE immunotherapy at a series of five (5) colon, three (3) pancreas cancer patients.

Surveillance of AHICE treatment was done at every two weeks following by a differential blood count, a lymphocyte immunophenotyping as wells as examining the cytokine-concentration of the TNF-α, IFN-γ, sIL-2R, sIL-6R in respect of those before AHICE-immune-stimulation. Generally, we have found:-Confluence with at first primary level of minimum > 1700 lymphocytes/μL peripheral blood and rising T4 in relation to T8-lymphocytes, an index of T4 to T8 better than 1.5, the TNF-α, IFN-γ, sIL-2R, sIL-6R cytokines were rising at a level of over 200% (TNF-α sIL-2R, sIL-6R) and over 110% (IFN-γ) concerning the therapeutical outcome-one colon-ca. patient overcomes the five years after surgery, without neoplasies/metastases until now, one other patient after surgery of liver-metastases, stage IV without further neoplasies until now, two (2) patients with liver-metas lived for 4 and 2 years (they undergone a combined chemotherapy/embolization). One patient with undefined Ca. (colon, ovarian or and peritoneal-ca.?, stage IV) have a remission of all neoplasies. One pancreas Ca. lived over nine years with best life quality and the shortest rest-over-life-time determined was 20 months.

In conclusion we can refer that after a previous de-masking of the tumor-cells, the so activated immune-system for autologous immunotherapy of tumors is the significant point of reference for successful-cancer treatment.

Chemokine Expression from Oncolytic Vaccinia Virus Enhances Vaccine Therapies of Cancer

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Tumor vaccines are capable of inducing robust immune responses targeting tumor antigens in the clinic, but anti-tumor effects have been disappointing. One reason for this is ineffective tumor infiltration of the cytotoxic T lymphocytes (CTLs) produced. Oncolytic viruses are capable of selectively replicating within tumor tissue and also inducing a strong immune response. We therefore sought to determine whether these therapies could be rationally combined such that modulation of the tumor microenvironment by the viral therapy could help direct beneficial CTLs induced by the vaccine into the tumor target. As such we examined the effects of expressing chemokines from oncolytic vaccinia virus, including CCL5 (RANTES), whose receptors are expressed on CTLs induced by vaccination with type-1-polarized DCs (DC1). We constructed and tested vvCCL5, an oncolytic vaccinia virus expressing CCL5. vvCCL5 induced chemotaxis of lymphocyte populations in vitro, and displayed improved safety in vivo. Interestingly, enhanced therapeutic benefits with vvCCL5 in vivo correlated with increased persistence of the viral agent exclusively within the tumor, the first time such an effect of expressing an immunomodulatory transgene from an oncolytic virus has been reported. However, vvCCL5 was also capable of attracting activated T cells into the tumor. When tumor-bearing mice were vaccinated with DC1 and then treated with vvCCL5 a further significant enhancement in tumor response was achieved which correlated with increased levels of certain tumor infiltrating lymphocytes. This approach represents a promising and novel means of applying combinations of biological therapies for the treatment of cancer.

Immunization of Disease-Free Melanoma Patients with Different HLA-A2 Peptides: Results of a Randomized Phase IB/II Trial Comparing Peptides Alone, Peptides in Montanide, Peptides + IMP321 (LAG-3Ig, An APC Activator), Peptides in Montanide + IMP321

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Design: We conduct a small open-label monocenter study. Twenty disease-free melanoma patients at high risk of relapse were randomized in 2006 to 2007. The first group of patients received 300 μg of each of the MAGE-1.A2, MAGE-3.A2, MAGE-4.A2, MAGE-10.A2, MAGE-C2.A2, NA17.A2, Tyrosinase.A2 and NY-ESO-1.A2 peptides, mixed together, without adjuvant. The

second group received the same 8 peptides emulsified in 1 mL of Montanide ISA51. The third group received the 8 peptides mixed with 200 μg of IMP321 (LAG-3Ig). The last group received the 8 peptides emulsified with Montanide and IMP321. These vaccines were administered every 3 weeks on 5 occasions by intradermal and subcutaneous injections. The disease status was assessed at study entry and thereafter every 3 months.

Results: Few side effects were reported with only grade 1-2 adverse events at injection sites. Immune responses were only seen in patients receiving a vaccine associating peptides and adjuvant. The median disease-free survival follow-up is now 33 months. Interestingly, all patients randomized in the peptides alone arm relapsed during the first year. In contrast, a majority of patients in the three other groups are still disease-free. Updated clinical and immunomonitoring data will be presented.

Conclusion: As expected, these vaccines were well tolerated. Antivaccine immune responses were observed only when an adjuvant was mixed with the peptides. Preliminary data suggest a clinical benefit for patients receiving a mix of peptides associated with an immunological adjuvant.

Two-Component Messenger RNA-Based Vaccines Provide Strong Anti-Tumoral Effect Especially in Combination with Radiation Therapy

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Complexation of mRNA with the cationic protein protamine generates two-component tumor vaccines with two principle activities: antigen expression and immune stimulation.

Compared to their single components, two-component mRNA vaccines induce superior innate as well as balanced adaptive immune responses: these comprise humoral as well as T cell mediated immunity and include induction of memory T cells. Immunization of mice bearing ovalbumin (Ova) positive E.G7 tumors with a two-component anti-Ova mRNA vaccine mediates a strong anti-tumor response also under therapeutic conditions. Anti-tumor efficacy depends on the size of established tumors at the beginning of treatment.

To test whether a combination of our vaccine with radiotherapy could achieve a therapeutic effect against large, clinical size tumors, mice were inoculated with E.G7 tumor cells and left untreated until the tumors reached a volume of around 200 to 250 mm^3. Mice were treated either with immunotherapy alone, radiation alone or combined radioimmunotherapy. Immunotherapy alone was only marginally effective against these large tumors, whereas radiation of the tumors induced transient growth stagnation for about 7 days. However, combined radioimmunotherapy dramatically improved anti-tumor efficacy. All mice treated this way showed pronounced tumor regression, causing complete and sustained eradication of the tumor in 3/7 mice. Median survival in the combination group was 45 days after start of treatment, compared to 9 days for untreated mice, 11 days for mice receiving immunotherapy and 17.5 days for mice in the radiation group.

These findings highlight that integration of immunotherapies with standard cancer therapies such as radiation creates highly synergistic anti-tumor effects, that may have the potential to enable long-term survival in cancer patients and ultimately to open a therapeutic avenue to cancer cure.

Peptide/IFA Emulsion Vaccines can Form a Sink and Graveyard for Tumor-Specific CD8+ T Cells

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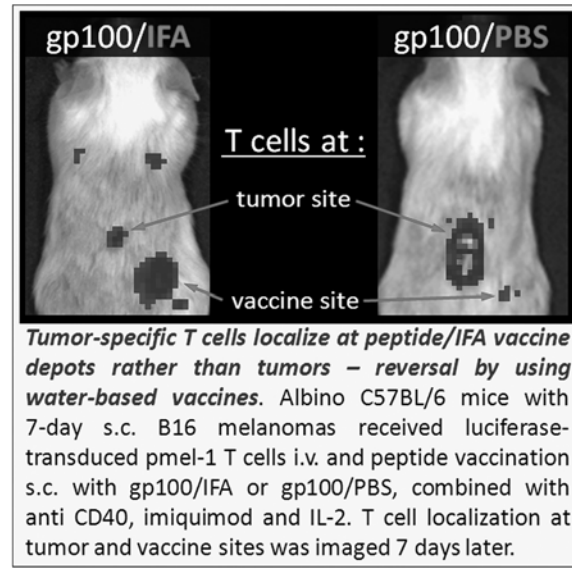

Tumor-specific T cells localize at peptide/IFA vaccine depots rather than tumors – reversal by using water-based vaccines. Albino C57BL/6 mice with 7-day s.c. B16 melanomas received luciferase-transduced pmel-1 T cells i.v. and peptide vaccination s.c. with gp100/IFA or gp100/PBS, combined with anti CD40, imiquimod and IL-2. T cell localization at tumor and vaccine sites was imaged 7 days later.

FIGURE 1.

Many current clinical cancer vaccine trials are based on minimal determinant peptides administered in vaccine vehicles that form stable depots, such as water-in-oil emulsions with Incomplete Freund's Adjuvant (IFA). Here we show that bioluminescent tumor antigen-specific CD8+ T cells preferentially localized to antigen-containing peptide/IFA vaccine depots rather than to antigen-positive tumors. Furthermore, T cells initially responded to peptide/IFA vaccination by proliferating but then rapidly disappeared without apparent memory formation. Peptide/IFA vaccination strongly prevented virus-induced T cell memory formation and destroyed pre-established T cell memory. This apparent tolerizing capacity of the peptide/IFA vaccine persisted for more than 30 days in vivo and correlated with chronic peptide antigen presentation in the vaccine-draining lymph node. Addition of CD40, TLR agonists, IL-2 or IL-23, while all boosting initial T cell response to peptide/IFA vaccination, did not prevent subsequent T cell tolerance. Peptide vaccination in saline, without IFA, failed to induce any T cell priming or tolerization, but peptide in saline with aCD40, TLR triggering and IL-2 support induced strong primary and secondary responses. Importantly, while IFA-based vaccines induced tumor-specific T cell localization at the vaccine site, water-based vaccines did not and instead resulted in T cell localization to the tumor site and superior anti-tumor activity. We propose that long-lived IFA vaccine depots such as currently used to vaccinate cancer patients can function as a sink and possibly graveyard for tumor-specific T cells, thereby limiting their therapeutic efficacy. Reducing vaccine depot half-life in vivo may result in more effective cancer vaccines (Fig. 1).

Host Immunity and Disease Free Survival are Improved in Breast Cancer Patients Receiving an Autologous Vaccine

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Introduction: To evaluate host lymphocyte immunity and disease free survival of breast cancer patients who were vaccinated with an autologous whole cell vaccine in the adjuvant setting.

Methods: We began preparing whole cell preparations for vaccine evaluation in 1995. Stage I and II breast cancer patients had host lymphocyte reactivity against tumor-associated antigens evaluated before and after treatment. Those patients with depressed immunity determined by a lymphocyte blastogenesis assay (LBA)

after standard adjuvant treatment were offered the autologous vaccine. Patients had six intradermal injections (three weekly and three monthly). Ten weeks after the last vaccine the LBA was repeated.
Results: Thirty-seven patients had autologous vaccines prepared and were vaccinated in the adjuvant setting. There were no severe toxicities and the vaccine was well tolerated with only slight pain and minimal swelling at the injection site. Patients have been followed for up to 15 years. Survival data of vaccinated patients with depressed immunity was compared to historic controls of unvaccinated patients with depressed and normal immunity to their tumor-associated antigens. The data suggests an improvement in overall survival of the vaccinated patients with depressed immunity.
Conclusion: The importance of maintaining good host lymphocyte immunity with cancer immunotherapy, after completion of conventional therapy, was confirmed by this study.

Immunotherapy of High Risk HPV Infections

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Therapeutic vaccination of persistent virus infections and associated diseases including (pre-) cancer so far has largely evaded success, mainly due to the fact that insufficiently consistent and robust effector T cell responses were induced by the commonly used vaccine constructs and formulations such as recombinant viruses and bacteria, recombinant proteins, DNA constructs, Dendritic Cell (DC) vaccines and exact HLA class I binding peptides (short peptides). Problems have been severe antigenic competion from vector sequences by recombinant viruses and bacteria, insufficiently powerful T cell generation by DNA constructs, insufficient homing to lymph nodes injected DC and antigen presentation of short peptides by non-professional antigen presenting cells in vivo, causing tolerance instead of immunity. Much more robust and consistent T cell responses can be obtained by vaccination with long (28-35 amino acid long) synthetic peptides. Such immunogens are more efficiently processed and presented than intact proteins by DC and only DC can efficiently perform this task. Moreover only concentrated antigen of choice is offered and antigenic competition therefore plays no role.
In earlier work we showed that therapeutic vaccination with a synthetic long peptide (SLP®) vaccine mediated the eradication of established human papilloma virus type 16 (HPV16)-positive tumors in mice and controlled wart growth and latent virus infection in rabbits persistently infected with cottontail rabbit papilloma virus. Subsequent phase I/II studies with an HPV16 SLP® vaccine, consisting of 13 long peptides covering the HPV16 E6 and E7 antigens, in patients with advanced HPV16-positive cervical cancer, revealed that this vaccine was safe and highly immunogenic. We then tested the clinical efficacy of this HPV16 SLP® vaccine in HPV16-induced high grade vulvar intraepithelial neoplasia (VIN3), a premalignant epithelial disorder, spontaneous regression of which occurs in less than 2% of patients and in which recurrence after standard treatment is high.
In a phase 2 trial, 20 women with VIN3 were vaccinated three times sc in the limbs with a mix of the HPV16 E6 and E7 synthetic long peptides formulated in Montanide ISA-51. The endpoints were objective clinical responses, defined as reduction of at least 50% in lesion size (partial response) or complete regressions, and HPV16-specific T cell responses. The vaccine was safe. At 3 and 12 months after the last vaccination an objective response was observed in 12/20 (60%) and 15/19 (79%) patients respectively. Nine of them showed a complete and durable regression of the lesions at 12 months and at 24 months. The strength of the vaccine-induced HPV16-specific T cell response was significantly higher in the group of patients with a complete regression of their lesions compared to non-responders. Patients with large lesions were less likely to experience a complete clinical response than patients with small lesions and we ascribe this to a larger proportion of vaccine induced HPV-specific regulatory cells in the patients with large lesions.
In conclusion, treatment with the HPV16 SLP vaccine is clearly effective in patients with established VIN disease. The SLP platform lends itself for development of therapeutic vaccines against many other chronic infections and non-viral cancers. In patients with cancer, it is attractive to combine this type of vaccination with immunogenic forms of cancer chemotherapy and with immuno-modulatory drugs.

Induction of CD8+ T Cell Responses Against Novel Glioma-Associated Antigen Peptides and Clinical Activity by Vaccinations With α-Type-1-Polarized Dendritic Cells and poly-ICLC In Patients with Recurrent Malignant Glioma

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Purpose: α-type-1-polarized dendritic cells (αDC1) are able to produce high levels of interleukin (IL)-12 and induce long-lived type-1 adaptive immune responses against tumor-associated antigens more efficiently than standard mature DC. A phase I/II trial was performed to evaluate the safety and immunogenicity of a novel vaccination with αDC1 loaded with synthetic peptides for glioma associated antigen (GAA) epitopes and administration of poly-ICLC in human leukocyte antigen (HLA)-A2+ patients with recurrent malignant gliomas. GAAs for these peptides are EphA2, IL-13 receptor (IL-13R)α2, YKL-40 and gp100.
Patients and Methods: Twenty-two patients [13 with glioblastoma multiforme (GBM), 5 anaplastic astrocytoma (AA), 3 anaplastic oligodendroglioma (AO) and 1 anaplastic oligoastrocytoma (AOA)] received at least one vaccination, and 19 patients received at least four vaccinations at two αDC1 dose levels ($1\times$ or $3\times$ 10^7/dose) at two-week intervals intranodally. Patients also received twice weekly intramuscular injections of 20 μg/kg poly-ICLC. Patients who demonstrated positive radiological response or stable disease without major adverse events were allowed to receive booster vaccines. T-lymphocyte responses against GAA epitopes were assessed by enzyme-linked immunosorbent spot and HLA-tetramer assays.
Results: The regimen was well tolerated. The first 4 vaccines induced positive immune responses against at least one of the vaccination-targeted GAAs in peripheral blood mononuclear cells in 11 of 19 (58%) patients. Booster vaccines induced positive responses in 4 additional patients. Analyses of peripheral blood demonstrated significant up-regulation of type-1 cytokines and chemokines, including IFN-γ and CXCL10. Eight (4 GBM, 1 AA, 2 AO and 1 AOA) achieved progression free status lasting at least 12 months. One patient with recurrent GBM demonstrated sustained complete response. IL-12 production levels by αDC1 positively correlated with progression-free survival.
Conclusion: These data support safety, immunogenicity and preliminary clinical activity of poly-ICLC-boosted αDC1-based vaccines.

Long Term Survival of a Rare Primary Renal Cancer Case Following Billateral Nefrectomy, Haemodialysis and Effective Final Impulse of Autologous Human Immune Vaccine Therapy

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Specified active Autologus Human Immune Cytokines Effectors (AHICE)- therapy against Cancer by treatment with the sum of the substances from the immune-mediator-cascade, which arise from patient's own blood, by autologus production. This autologous

immune response eliminate everywhere, the patient's body, the recognised none histocompatible cancer cells through his unique selectivity, detection-ability intelligence.
We present a rare case of a 70 year old man with primary renal adenacarcinoma. The first diagnosis was fifteen years ago and was followed by right nefrectomy. The follow-up study was discovered a remission in the left kidney three years later. The patient was undertaken in a partial left nefrectomy but one year later a new one mass in the left renal remnant was revealed by follow up research study and a complete bilateral nefrectomy was the final surgical curative result. Because of the renal failure he was under haemodialysis the last 11 years. Chemotherapy by antiangiogenetic regimen was followed because of liver secondaries without improvement. Two years ago applied in a 2-cycle simulation AHICE-therapy with successful results and remission of liver secondaries. The patient died, 10 months ago, suddenly, during his gardening usual activities from a heart infarct.
In conclusion we address the effectiveness of impulse AHICE-therapy in a rare, difficult and immunocompromised case of an elderly man.

Autologous Human Vaccine, AHICE, for Multi-Primary Cancer Therapy

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The Autologous Human Immune Vaccine AHICE therapy is of high specificity against tumour- and their metastatic cells. Their autologous production is initiated on at a specific biochemical treatment (patent). This means, that patient's own immune-system will get the specific information of the altered shape of the target-, cancer- cells and is thereafter able to identify them as none healthy body-cells. After that, the immune-system begins to kill the so identified target cells via antigen-specific activation of the natural killer cells (NKC). This is highly specific, but very complexed immunological/biochemical mechanism. Thereafter, it is the greatest benefit, that patient's own immune-system can so identify and eliminate the altered target cells, not only in the primary tumour site, but also the metastatic regions everywhere in the whole patient's body (''intelligent phenomenon''). In this consecutive case series study we report the therapy principles and results of thirty (30) multi-primary cancer patients. In five cases a second one AHICE cycle was followed. The period of each one therapy cycle was 90 days. No one patient had side effects or complications. Two patients presented an ischemic brain attack and ascites, without any relation to vaccine therapy.In conclusion, analysis of our present study results refers that lymphocytes are the most important immune component for active immune anticancer management. AHICE therapy is well tolerated, effective, uncomplicated plus good quality of life and survival anticancer optional therapy. We need more cases for randomised trials in the near future.

Adoptive Immunotherapy for Recurrent Ovarian Cancer Using Autologous Whole Tumor Antigen-Primed T Lymphocytes

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Background: Novel therapeutic strategies are warranted in recurrent ovarian cancer. We report the pilot application of combinatorial immunotherapy comprising dendritic cell (DC)-based autologous whole tumor antigen vaccination in combination with antiangiogenesis therapy, followed by adoptive transfer of autologous vaccine-primed CD3/CD28-costimulated lymphocytes in patients with recurrent ovarian cancer.
Methods: Patients with recurrent progressive stage III and IV ovarian cancer underwent priming with intravenous bevacizumab and oral metronomic cyclophosphamide, followed by vaccination with DCVax-L, an autologous DC preparation pulsed with autologous tumor lysate, plus bevacizumab. This was followed by lymphodepletion using high-dose outpatient cyclophosphamide and fludarabine (cy/flu) and transfer of autologous vaccine-primed, ex vivo CD3/CD28-costimulated peripheral blood T cells, in combination with DCVax-L vaccination.
Results: Six subjects received vaccination alone; three subsequently completed T cell transfer therapy. Vaccination with DCVax-L following bev/cy produced few grade 1 toxicities and elicited tumor-specific T cell and humoral responses. Partial objective response was observed in two patients after completion of vaccine; two additional patients demonstrated stable disease. Among the former, one subject progressed through bev/cy, but had objective response to DCVax-L. Three vaccine recipients with detectable vaccine priming subsequently received adoptive transfer of vaccine-primed, CD3/CD28-costimulated autologous T cells following outpatient cy/flu lymphodepletion. One patient, who exhibited no evidence of disease at end of study, experienced a durable reduction of CD4 + FOXP3 + T regulatory cells, increased total lymphocyte counts and restoration of vaccine-induced antitumor immunity. Stable disease was observed in a second subject. In the third subject, adoptive immunotherapy was not followed by restoration of vaccine-induced antitumor immunity and disease progression was observed. Updates will be presented at the meeting.
Interpretation: The use of combinatorial cellular immunotherapy comprising bev/cy with DC vaccination with whole tumor antigen and adoptive immunotherapy using tumor antigen-primed T cells for the treatment of patients with recurrent ovarian cancer warrants further investigation.

T Cell Activation, PSMA Seroconversion and Increased Th17 Rates are Associated with Favorable Clinical Outcome in Prostate Cancer Patients Treated with Prostate GVAX and Anti-CTLA4 Immunotherapy

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The effects of Prostate GVAX and the anti-CTLA4 antibody Ipilimumab were investigated in a Phase I dose escalation/expansion trial of patients with prostate cancer. Results showed that the GVAX/Ipilimumab combination was clinically active with PSA declines of more than 50% (Partial Response, PR) in 5 of 22 patients and PSA stabilizations (Stable Disease, SD) in 7 of 22 patients in the higher (3 to 5 mg/kg) Ipilimumab dose levels. Immune response monitoring was performed to identify changes that might predict or correlate with clinical efficacy. Most notably, pronounced and significant increases in frequencies of activated CD4 + and CD8 + T cells were observed by HLA-DR and ICOS expression upon administration of high (3-5 mg/kg) but not of low (0.3 to 1 mg/kg) Ipilimumab dosages. Of these, only early HLA-DR up-regulation might be useful as a marker for response prediction, since it was observed to significant levels in PR or SD, but not in PD patients. As an indication of tumor-specific responsiveness we tested NY-ESO-1 and PSMA specific seroreactivity and HLA-Tetramer (Tm) binding. For NY-ESO-1, GVAX/Ipilimumab-induced increased seroreactivity was observed in 6/28 patients. In 2 of 3 of these patients, increased NY-ESO157 T cell rates were also found, whereas no Tm reactivity was detected in 8 patients without NY-ESO-1 seroreactivity. Interestingly, PSMA seroconversions were observed in a total of 12/28 patients and were found to

associate with improved overall survival. However, so far no PSMA Tm positivity was found. In addition, GVAX/Ipilimumab administration was found to induce Th2/Th17 profiles, as determined ex vivo by intracellular staining of peripheral T cells. Significantly increased levels of IL-4 in both CD4+ and CD8+ T cells were observed in patients with PR or SD, but not in patients with PD. Of note, Th17 spikes in 3/5 patients coincided with autoimmune breakthrough events and PSA responses. In summary, our data show that PSMA seroconversion, early HLA-DR up-regulation on T cells and increases in Th17 rates are associated with a favorable clinical outcome. Together these data point to a mechanism of action whereby combined Prostate GVAX and anti-CTLA4 immunotherapy can induce both Th2/humoral and Th17/cell-mediated immune responses, resulting in tumor destruction and collateral autoimmunity.

Vaccine Induced Increase of CCR7+ CD8+ T Cells is Associated with Favorable Clinical Outcome in a Wilms Tumor Gene 1 (WT1) Peptide- Vaccination Trial in Leukemia Patients

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Objectives: In the context of a phase I/II study with WT1-peptide-vaccination in AML patients we could previously demonstrate immunological and clinical responses but did not observe a correlation between T cell responses and outcome parameters in the initial analyses of tetramer staining and intracellular cytokine staining for TNF alpha and IFN gamma. We here report on more detailed immunological analyses in a subgroup of patients, leading to the identification of an increased percentage of CCR7+ CD8+ memory T cells in the peripheral blood (PB) as a beneficial phenotypic marker for WT1 peptide vaccine efficacy.

Methods: After vaccination with 62.5 µg GM-CSF (day 1 to 4) and 0.2 mg HLA-A0201-restricted WT1.126 to 134 peptide (day 3) admixed with 1 mg KLH 16 HLA-A2-positive AML patients were available for detailed immunological analyses. Peripheral blood mononuclear cells (PBMC) at baseline and 10 weeks after vaccination start were analysed by multicolour flow cytometry for phenotypic and functional characteristics. In detail CCR7 expression, induction of PD1 and CD137, and the capacity to produce IFN-y, TNF-a and IL-2 and mobilize CD107a, in response to the WT1 vaccine peptide were evaluated ex vivo. T cell response was correlated with time to treatment failure (TTF).

Results: In neither WT1 specific CD3+ CD8+ T cells nor in the whole CD3+ CD8+T cell population a significant correlation could be found between cytokine or cytotoxic response, the activation marker CD137 or the exhaustion marker PD-1 and TTF. Interestingly, we observed a significant increase of CCR7+ CD8+ T cells in patients with favourable clinical course. Patients with an increase of CCR7+ CD8+ T cell frequencies (46.4% week 0 to 67.4% week 10) during vaccination had a significantly ($P < 0.01$) longer TTF (median = 407 d, range 128 to 1200 d, n = 8) than patients with a decrease of CCR7+ CD8+ T cells (33.1% week 0 to 26.6% week 10) and a shorter TTF (median = 137 d, range 57 to 201 d, n = 8).

Conclusion: Our results indicate that the vaccine-induced increase in CCR7+ CD8+ memory T cells may represent a surrogate marker for clinical efficacy in vaccination and warrant evaluation in future clinical trials.

Vaccine Combinations: Endogenous Versus Exogenous Vaccination with CTLA-4 Blockade

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Blockade of immunologic checkpoints results in durable regressions in a variety of malignancies. Specifically, the use of the CTLA-4 blocking antibody, ipilimumab (ipi) results in improved overall survival in patients with refractory melanoma. Other agents, such as MDX-1106 an antibody blocking PD-1 also mediate durable responses in melanoma, renal cell carcinoma and non-small cell lung cancer. Important questions include identification of targets for antigen-specific immunity which are mechanistically linked to clinical benefit and the role for vaccination in enriching the population of patients who have clinical benefit. The results of a recently completed phase III randomized trial comparing ipi with or without HLA-A*0201 restricted gp100 peptides or gp100 alone in patients with refractory melanoma show that the ipi containing groups have improved overall survival compared with peptides alone. The use of peptides with ipi did not improve the overall survival and, interestingly, resulted in slightly inferior radiographic response rates and progression-free survival. One hypothetical explanation for these results is that the introduction of a small number of antigens from one protein (gp100) during the time of immune potentiation biases the disinhibited immune response to a repertoire skewed to those specific peptides, which may or may not be relevsnt to all tumors in all patients. Eventually, the broad immune potentiation mediated by ipi allows for the amplification of responses to other antigens as overall survival is not affected by the use of peptides. The data from this study are even more intriguing when put in the context of results from a trial using high-dose bolus IL-2 with or without gp100 peptide, which showed an improvement in progression free survival and response rate with the combination, compared with IL-2 alone, but no change in overall survival.

In considering how to best build on these results when designing clinical trials using immune modulators and vaccines, it is best to consider the differences between exogenous and endogenous vaccination. We, and others, have investigated antigens recognized by patients treated with ipi and have found that those who have pre-existing or induced immunity to NY-ESO-1 are more likely to achieve durable disease control. The mechanistic versus surrogate role of immunity to this particular antigen is remains a question; nonetheless, it is instructive to learn that patients have spontaneous immune responses to this protein which may be broadened (more epitopes) or improved (addition of CD8+ polyfunctional T cells to an existing antibody or CD4+ response) after CTLA-4 blockade. Additional ways to improve clinical response need careful consideration. Single epitope antigens present the risk for the emergence of escape variants, so the use of proteins, whole cells or even cellular libraries of antigens (such as heat shock protein-96 conjugated peptides) are important considerations for future combination trials. Timing of administration of vaccine may also be critical as expansion of cells already primed in the repertoire by vaccination in the past may be advantageous compared with concurrent vaccination. Finally, the use of endogenous means of vaccination should be considered. Necrotic or apoptotic tumor is a source of antigen for patients responding to immunotherapy. Therefore, local tumor destruction and rational inclusion of systemic therapies with immune modulation are obvious next-steps. Systemic therapies could include either chemotherapies which now have well-described immunologic effects as well as signal transduction pathway inhibitors which also possess the ability to arrest tumor growth in appropriately genotyped patients.

Therapeutic Vaccination Targeting Indoleamine-2, 3 Dioxygenase (IDO) Using a IDO Derived Class I Restricted Peptide in Combination with Aldara and Montanide for Patients With Locally Advanced or Metastatic Non Small-Cell Lung Cancer. A First-in-man Phase I Trial

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Background: Non small-cell lung cancer (NSCLC) is a common disease with a poor prognosis when locally advanced or metastasized, despite advances in surgery, chemo- and radiation therapy.

Indoleamine 2, 3-dioxygenase (IDO) is an immunoregulatory enzyme that are implicated in suppressing T cell immunity in normal and pathological settings. Expression of IDO has been shown to induce T cell anergy and/or the generation of adaptive regulatory T cells. In cancer patients IDO is expressed within the tumor itself as well as in antigen-presenting cells in tumor-draining lymph nodes, where it promotes the establishment of peripheral immune tolerance to tumor antigens. Recently, we showed that IDO derived peptides are subject to cellular immune responses. Hence, cytotoxic T cell reactivity against the IDO derived peptide IDO5 (sequence) restricted by HLA-A02.01 are present in peripheral blood as well as in the tumor microenvironment of cancer patients. We demonstrated that these IDO reactive T cells are indeed peptide specific, cytotoxic effector cells. IDO reactive T cells are able to recognize and kill tumor cells including directly isolated AML blasts as well as IDO-expressing dendritic cells, that is one of the major immune suppressive cell populations. Consequently, IDO may serve as an important and widely applicable target for anti-cancer immunotherapeutic strategies.

Methods: To test this notion fourteen patients with incurable stage III-IV NSCLC off chemotherapy is included in this study. Further inclusion criteria: PS (0-1), at least one measurable lesion in CT-scan, and acceptable laboratory values. Patients are administered bi-weekly IDO vaccinations subcutaneously, and undergo CT-scan evaluation after 6 vaccinations. In the absence of progression, patients receive monthly vaccination thereafter until progression. Primary end points are safety and toxicity. Secondary end points are immunological and clinical response.
Results: The study is approved by the Danish health authorities and registered at www.clinicaltrials.gow, and patient inclusion is ongoing. Preliminary data on toxicity and immune parametres will be presented.
Conclusion: We assess vaccination against the IDO enzyme as a potential target for anticancer treatment. IDO is expressed in cancer cells and antigen presenting cells; and the vaccination against IDO expressing cells is therefore two-sided. Safety and toxicity are primary goals.