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Poster Information

Poster Hall Location
Main Level, Grand Ballroom, Salons A – D

Poster Set-Up Hours
Friday, November 4 from 6:00 am – 10:00 am

Poster Tear Down Hours
Saturday, November 5 from 8:00 pm – 9:30 pm

Poster Hall Hours
Friday, November 4 from 10:00 am – 8:00 pm
Saturday, November 5 from 10:00 am – 8:00 pm

Poster Presentations
Odd Number Posters (authors are present)
Friday, November 4 from 12:30 pm – 1:30 pm & 6:15 pm – 7:00 pm

Even Number Posters (authors are present)
Friday, November 4 from 7:15 pm – 8:00 pm
Saturday, November 5 from 12:30 pm – 1:30 pm

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COMBINED USE OF POLARIZED AND NON-POLARIZED DCs FACILITATES ACCELERATED GENERATION OF HIGH NUMBERS OF Ag-SPECIFIC CTLs

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Effective induction of high numbers of tumor-specific $CD8^+$ T cells requires several booster doses of vaccines. In an attempt to shorten the minimal interval between prime and booster doses of immunotherapy, we evaluated the interplay between different subsets of DCs with different abilities to induce effector functions in tumor-specific $CD8^+$ T cells.

We show that priming of naïve $CD8^+$ T cells by Ag-loaded DCs matured in the presence of prostaglandin E2 (PGE_2; non-polarized DC) induce the accelerated differentiation of naïve $CD8^+$ T cells into cells with a central-memory phenotype ($CCR7^+IL\text{-}7R^+GrB^{low}$) without passing through a cytolytic effector phase. In contrast, naïve $CD8^+$ T cells primed by DCs matured in the presence of TLR-ligands and interferons (polarized DC1s) differentiate into effector-type CTLs ($CCR7^-IL\text{-}7R^-GrB^{high}$).

The non-cytolytic $CD8^+$ T cells induced by the non-polarized DCs rapidly acquire CTL function upon secondary interaction with polarized DC1s. Such non-polarized DC-primed $CD8^+$ T cells can respond to secondary stimulation with Ag-loaded DC1s with secondary expansion much sooner than the polarized DC1-primed $CD8^+$ T cells (CTLs), and avoid activation-induced apoptotic cell death at early time points after activation. Analysis of the RNA expression showed that the non-polarized DC-primed $CD8^+$ T cells have the same gene expression pattern as blood-isolated central-memory T cells.

The selective ability of non-polarized DCs to induce the differentiation of naïve $CD8^+$ T cells into central-memory cells, which are able to rapidly expand and acquire effector function upon restimulation with polarized DC1s, may allow to shorten the time between prime-boost vaccinations, accelerate the generation of high numbers of Ag-specific effector $CD8^+$ T cells, and provide for new means of enhancing the effectiveness of current immunotherapies of cancer.

Key Words: CD8+ T cells, Dendritic cell.

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SOURCING HUMAN BLOOD-DERIVED RAW MATERIAL: OPTIMIZATION, QUALIFICATION AND CONTROL

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Human cells and tissue are critical raw material for cell therapy and tissue-engineered products, as well as ex vivo gene therapy products. Quality of this living cellular raw material is a major determinant of final product characteristics, but is complicated by the inherent heterogeneity and inter-individual variability of living biologics. Inconsistent collection procedures amplify this biological variability, adversely affecting the manufacturing process from the outset. Controlling and qualifying the cell collection step is essential to minimizing operational sources of variability, and greatly increases the likelihood of success in manufacturing. HemaCare's core competency is apheresis collection, and, building on 34 years of experience, has addressed this need for optimized cell collection with a program for controlling and qualifying its apheresis procedures and collection sites, to supply human-derived blood components for development and qualification of novel cell and gene therapies, assays, and medical devices. HemaCare's apheresis program includes comprehensive staff qualification and training, documentation that supports its cGMP environment and programs to monitor effectiveness of equipment and procedures in accordance with an established quality system. Donor recruitment, screening and IRB-approved consents follow the requirements of Good Tissue Practices (GTPs). From 2006-2010, inclusive, a total of 55,262 apheresis procedures were performed, including collection of patient and normal-donor peripheral blood mononuclear cells, mobilized peripheral blood progenitor cells, plateletpheresis products, and therapeutic apheresis. In 2010 alone, 11,646 apheresis products were collected, many of which supported clinical studies spanning Phase I - Phase III, preclinical research, and, for the first time, commercial cell therapy applications. This reflects the evolution of cellular therapies.

Key Words: Immunotherapy, apheresis collection.

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DENDRITIC CELL DIFFERENTIATED WITH 15 KD GRANULYSIN: A REPLACEMENT FOR GMCSF?

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Granulysin is an antimicrobial and proinflammatory protein with several isoforms. The 9 kDa isoform has proinflammatory, chemotactic and cytotoxic function against microbes and tumors. The 15 kDa isoform is functionally not well characterized although has been shown to act as an alarmin and able to activate monocytes and dendritic cells (DCs). We have shown with gene expression analysis that monocytes stimulated with 15 kDa Granulysin or GM-CSF share a common gene signature, but only 15 kDa Granulsyin was able to induce activation of pathways related to fundamental dendritic cell functions. Therefore we decided to investigate the possible use of 15 kDa Granulysin for the production of dendritic cells (GNLY-DCs) as alternative to the current process which involves the use of GM-CSF (GMCSF-DCs). We analyzed gene expression profiles and cytokines production of immature DCs (iDCs) obtained from elutriated monocytes of 5 different healthy donors, differentiated for 3 days in presence of IL-4 (1000 IU/ml) and one of the following: 15 kDa Granulysin (10 nM), GMCSF (1000 IU/mL), GMCSF (56 IU/mL). Both analyses were repeated after maturation using IFNγ (1000 IU/mL) and LPS (30ng/mL). Independently of the protocol used, iDCs showed no statistical differences in both gene expression profile and cytokine secretion. Mature GNLY-DCs express higher levels of several key immune genes as revealed by gene expression profiling. Cytokine secretion analysis showed that GMCSF-DCs secreted higher amounts of IL-6, TNFα and MCP1 (CCL2), whereas IL-12, IL-8, IP10, MIP3α, MDC, MIG did not show statistical significant differences. No differences were detected between high and low dose GMCSF-DCs. In conclusion, GNLY-DCs share most of the features with GMCSF-DCs, but possess also some promising features, suggesting that GNLY-DCs might be tested in future immunotherapies aimed to activate the immune response.

Key Words: Dendritic cell.

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AUTOPHAGY INDUCED BY INTERFERON-GAMMA IN MELANOMA CELLS IS ASSOCIATED WITH BETTER CLINICAL OUTCOMES IN PATIENTS RECEIVING CELL-BASED IMMUNOTHERAPY AGAINST MELANOMA

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BACKGROUND & RATIONALE The use of either necrotic or apoptotic cells in whole-cell immunotherapy protocols continues to be controversial. Interferon-gamma (IFN-g), a pleiotropic cytokine, has been shown to induce both apoptosis and autophagy in some cell types. Autophagy is a homeostatic process by which cytosolic components and organelles are delivered to the lysosome for degradation. Autophagy is also associated with innate and adaptive immune responses to intracellular pathogens whereby cytosolic antigens are loaded onto major histocompatibility complex (MHC) class II molecules for CD4+ T-cell recognition. We previously noted a correlation between the presence of apoptotic cells and poor survival in a clinical trial involving the use of IFN-g treated autologous melanoma cells loaded onto dendritic cells for use in cell-based immunotherapy vaccine. In the current series of studies, we investigated the induction of autophagy, apoptosis and MHC class II molecules after IFN-g treatment of melanoma tumor cells in vitro. METHODS Autologous and model melanoma tumor cell lines were incubated with 1000 IU/mL of IFN-g for 72 hours prior to assaying for autophagy, apoptosis and MHC class II expression. Autophagy was detected by immunobloting with antibodies against LC3 II and by flowcytometry with Enzo's CytoID Autophagy Detection Kit. Apoptosis and MHC class II induction were assayed by flowcytometry using 7-AAD and annexin-V staining and antibodies against MHC class II, respectively. RESULTS The results demonstrated that IFN-g induces both autophagic and apoptotic cell populations in melanoma cell lines. The apoptotic population is predominantly found in the non-adherent population while the autophagic cells remain adherent to the flask. Blocking of autophagy with the inhibitor 3-methyladenine (3-MA) inhibits the induction of MHC class II positive cells in response to IFN-g (39.4% IFN-g vs. 10.0% IFN-g + 3-MA). Inhibition of caspase activity with the pan caspase inhibitor Z-VAD prevents apoptosis but does not perturb autophagy in IFN-g treated cells (2.75 ± 0.15 IFN-g vs. 3.04 ± 0.27 IFN-g + Z-VAD, fold change). CONCLUSIONS Induction of apoptosis is associated with reduced levels of autophagy and MHC class II induction. A simple procedure to eliminate apoptotic cells while retaining viable autophagic cells after IFN-g treatment may enhance the effectiveness of this type of cell-based immunotherapy.

Key Words: Apoptosis, Dendritic cell, Melanoma.

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MELANOMA PATIENTS TREATED WITH DENDRITIC CELL VACCINATION, INTERLEUKIN-2 AND METRONOMIC CYCLOPHOSPHAMIDE - RESULTS FROM A PHASE II TRIAL

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Background: Dendritic cells (DC) are the most potent antigen presenting cells and have proven effective in stimulation of specific immune responses in vivo. Despite of this, DC vaccination studies have yet to prove clinical effective. In this phase II DC vaccination trial metronomic cyclophosphamide (Cy) and a cox-2 inhibitor have been added with the intend to overcome immunosuppressive mechanisms.

Methods: 28 patients with progressive metastatic melanoma were included. Vaccines consisted of autologues DCs pulsed with survivin, hTERT and p53 derived peptides (HLA-A2+) or tumor lysate (HLA-A2-). Vaccine no. 1-4 was given weekly, vaccine 5-10 biweekly and monthly vaccines thereafter until disease progression. Concomitantly the patient was treated with IL-2, 2 MIU for five days following each vaccine, Cy 50 mg twice a day every second week and Celecoxib 200 mg daily.

Primary objectives were safety and tolerability, secondarily immunological and clinical responses, time to progression (TTP) and overall survival (OS) were assessed.

Results: The treatment was safe and tolerable. 16 patients (57%) had SD according to RECIST at 1st evaluation and 8 patients had prolonged SD (7-13.7 months). The median TTP was 4.5 months and the median OS was 9 months. Patients with SD had a median OS at 13.7 months; patients with progressive disease (PD) had an OS at 6.0 months ($p = 0.02$). There were no differences in prognostic factors between the two groups.

Despite the use of metronomic Cy blood level of regulatory T cells (Treg) did not decrease during treatment.

Indirect IFN-γ ELISpot assays have shown induction or increase of antigen specific immune responses in 11 out of 15 screened HLA-A2+ patients. In particular responses against telomerase peptides (9 patients) have been seen but also survivin (3 patients) and p53 (2 patients) responses were observed.

Conclusion: The number of patients obtaining SD more than doubled compared to a previous trial without Cy and patients with SD lived significantly longer than patients who had PD after 8 weeks. 11 of the 15 screened HLA-A2+ patients had an increase or an induction in immune responses against the tested peptides. Notably, metronomic Cy was unable to reduce Treg level.

Key Words: Cancer vaccine, Dendritic cell, Treg cells.

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RNADJUVANT, A NOVEL HIGHLY POTENT RNA-BASED ADJUVANT SUPPORTS INDUCTION OF BALANCED IMMUNE RESPONSE (TH1 AND TH2) AND ANTI-TUMOR ACTIVITY

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For efficient cancer immunotherapy, strong adjuvants are mandatory to induce a potent and persistent immune response, particularly as tumor associated antigens used as purified proteins or peptides show mostly only a weak intrinsic immunogenicity. CureVac have recently developed a novel RNA-based adjuvant with strong immunostimulatory properties. RNAdjuvant is physically and chemically well-defined with a very good safety profile. RNAdjuvant is well tolerated even at high doses and does not induce in mice the splenomegaly described for standard adjuvants such as CpG-DNA. RNAdjuvant supports both the antigenic formats of recombinant proteins and peptides, causing dramatically increased immunogenicity of these vaccines, especially in respect of T cell responses and anti-tumor activity.

Mode of action studies demonstrate that the multilateral function of RNAdjuvant which positively affects the quality and quantity of innate and adaptive immune response.

In vitro studies in human peripheral blood monocyte cells (PBMC) showed that RNAdjuvant is preferentially taken up by antigen presenting cells (APC). Uptake of RNAdjuvant is accompanied by strong activation of APC reflected by increased expression of specific activation markers and secretion of cytokines driving a pronounced TH1 response. Moreover, RNAdjuvant stimulates the innate immune response by secretion of IL-12 and activation of (natural killer) NK cells. Vaccination with recombinant Ovalbumin in combination with RNAdjuvant , elicited strong antigen-specific cytotoxic T-cell responses, which are barely induced by vaccination with recombinant ovalbumin alone. Prophylactic vaccination with RNAdjuvant-enhanced ovalbumin mediated a complete protection against Ovalbumin expressing tumors, whereas vaccination with Ovalbumin alone had no impact on tumor growth. Moreover, RNAdjuvant also enhanced the humoral response strongly supporting the shift towards TH1 response as determined by increased titers of IgG2a antibodies.

Peptide-based vaccines, including Ovalbumin-derived SIINFEKL epitope and Human Papillomavirus (HPV)-derived peptides, also strongly benefited from RNAdjuvant and exhibited significantly higher T cell responses compared to non-adjuvanted counterparts or adjuvanted with CpG-DNA. Interestingly, exclusive induction of SIINFEKL specific CD8+ T cells, in mice vaccinated with adjuvanted SIINFEKL peptide, was completely insufficient to provide any anti-tumor activity.

Taken together our data demonstrate that RNAdjuvant represents a novel breakthrough technology which may revolutionize the field of cancer vaccines requiring safe and potent adjuvants.

Key Words: Adjuvant, Cancer vaccine, Dendritic cell.

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TUMOR CELLS CONTAIN 'VETO' FACTORS LIMITING IMMUNOGENICITY OF STERILE NECROSIS AND CONTROLLING CD8+ T CELL ACTIVATION

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CD8+ T cell activation and generation of effector immune response is important for anti-tumor immunity as well as for successful anti-cancer vaccination and therapy. Therefore dissecting mechanisms, which lead to the induction or suppression of the function of these cells as a consequence of tumor cell death, is of great interest. We have investigated the cellular and molecular events responsible for T cell activation during sterile necrotic cell death in the absence of Pathogen Associated Molecular Patterns (PAMPs).

Sterile necrosis was induced by 3 freeze/thaw cycles in Ovalbumin (OVA) expressing, MHC class I negative tumor cells. Sterile necrotic cells failed to stimulate CD8+ T cell response when incubated with dendritic cells (DCs) in vitro. In contrast, activation of 3xF/T necrotic cells reversed them into an immunogenic state and induced proliferation and IFN-γ secretion by antigen-specific CD8+ T cells in vitro.

Injection of immunogenic necrotic cells in vivo or adoptive transfer of CD11c+ splenic DCs loaded with immunogenic necrotic cells led to efficient adaptive immune response and protection from tumor challenge. In contrast non-immunogenic necrotic (3xF/T) tumor cells failed to induce OVA specific CD8+ T cell dependent immunity.

We hypothesized and now show that 3xF/T tumor cells contain certain factors, which limit antigen processing/presentation and therefore make necrotic cells non-immunogenic. We refer to these as 'veto' factors.

Using classical chromatography and mass-spectrometry approaches, we have identified two different types of 'veto' factors. When released from 3xF/T necrotic cells they can abort priming of CD8+ T cells. Addition of exogenous 'veto' factors to in vitro culture of DCs and T cells drastically reduced proliferation and IFN-γ secretion from antigen-specific T cells.

In vivo co-administration of exogenous 'veto' factors together with immunogenic necrotic cells also precluded the generation of antigen-specific immune response.

These results demonstrate that impairment of immunogenicity of dead cells in conditions when cellular integrity is lost and intracellular content is released, is a novel mechanism to control adaptive immune responses and these findings will help in designing better cancer vaccines.

Key Words: Cancer vaccine, CD8+ T cells, Dendritic cell.

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IMMUNOGLOBULIN-LIKE TRANSCRIPT (ILT) RECEPTORS ON HUMAN DERMAL CD14+ DCS ACT AS A CD8-ANTAGONIST TO PREVENT EFFICIENT CTL PRIMING

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Human Langerhans Cells (LCs) are highly efficient at priming naïve CD8+ T cells into potent CTLs. In comparison, dermal CD14+ DCs could initiate weaker CD8+ T cell responses with low granzymes A, B and perforin and a limited capacity to kill target cells.

Here we show that dermal CD14+ DCs give rise to a population of CD8+ T cells sharing the property of Type2-cytokine secreting CD8+ T cells (TC2) that express low levels of CD8, secrete type 2-associated cytokines including IL-10, and express the TC2-associated markers CD40L and CD30.

Addition of anti-CD8 antibodies to co-cultures of LC and naïve CD8+ T cells results in the induction of TC2 cells suggesting the importance of CD8 co-signals for the priming of highly efficient CTLs. The Immunoglobulin-Like Transcript (ILT) inhibitory receptors are expressed on dermal CD14+ DCs and act as CD8-antagonists. Indeed, soluble-ILT-2 and ILT-4 could inhibit effector CD8+ T cell priming by LCs, together with promoting IL-4 and IL-10 production. Conversely, blocking ILT-2 or ILT-4 on dermal CD14+ DCs enhanced the generation of effector polyfunctional CD8+ T cells.

Thus, our results strongly suggest that ILT receptor family members can control the polarization of CD8+ T cell responses, and manipulation of these receptors might have great implications in tumor immunotherapy.

Key Words: CD8+ T cells, Dendritic cell.

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FACILE GENERATION OF APCS USING SOLUBLE MULTIMERIC CD40L AS A B CELL PROLIFERATION STIMULUS

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Human B cells proliferate massively ex vivo when treated with a CD40 stimulus and IL-4. Such "CD40-B cells" are known to be strong antigen-presenting cells (APCs) that can replace dendritic cells (DCs) for many applications. However, the production of CD40-B cells generally requires co-culture with cells lines that express membrane CD40L, since this form of CD40L supplies a many-trimer stimulus needed to cluster CD40 receptors and

thereby activate the B cells. This added level of complexity has compromised the practicality of using CD40-B cells instead of DCs for generating anti-tumor CD8+ T cells. To circumvent this problem, we tested a 4-trimer, multimeric soluble form of CD40L produced by fusing the body of surfactant protein D with the extracellular domain of CD40L to make SP-D-CD40L (UltraCD40L™). This protein was produced by CHO cells and is stable for several weeks at room temperature and > 5 months at 4 oC. To grow CD40-B cells, CD19+ or CD20+ cells were immunomagnetically selected from venous blood and cultured with 10-20% CHO cell supernatant plus IL-4. About 100-fold expansion of B cells occurred in two weeks and the resulting cells were >95% positive for CD19, CD80, and CD86. These growing CD40-B cells can be cryopreserved and then thawed for further expansion many months later. To use these CD40-B cells as APCs, they were treated with mitomycin and pulsed with the NLV peptide, a prototypic antigen from CMV pp65 that is an immunodominant epitope for HLA-A2.1 donors. Using these cells as APCs for either PBMCs or purified CD8+ T cells, antigen-specific CD8+ T cells were generated that were recognized by NLV/A2.1- tetramer staining. By using UltraCD40L™, this advanced CD40-B cell system provides a facile way to generate proliferating APCs that should prove very useful for the active immunotherapy of cancer.

Key Words: B cell, CD8+ T cells, Dendritic cell.

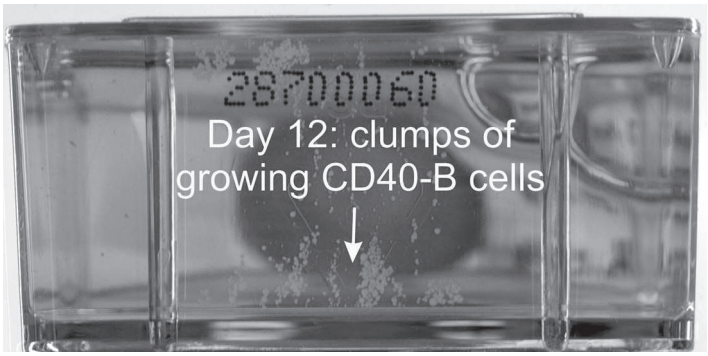

Figure 1. Starting with a single tube of blood, B cells can be grown using UltraCD40L + IL-4 to about 100 million cells in less than two weeks. These highly activated cells express high levels of MHC-I, CD80, and CD86, and can be used to generate antigen-specific CD8+ T cells.

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HUMAN DENDRITIC CELLS DIFFERENTIATED IN THE PRESENCE OF ADENOSINE RECEPTOR AGONISTS DISPLAY A TOLEROGENIC PHENOTYPE AND FAIL TO PRIME CD8 T CELLS

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Mammalian organisms have evolved multiple endogenous mechanisms that serve to regulate immune and inflammatory responses in steady-state conditions or upon resolution of the acute phase of infections or injuries. These safeguards ensure the maintenance of peripheral tolerance and prevent the establishment of chronic inflammation. However, it is becoming increasingly appreciated that tumors are able to exploit these homeostatic processes in order to subvert and evade immune responses. Recent investigations have pointed to the generation of adenosine in the tumor micro-environment as one such double-edged regulatory mechanism. Notably it was shown that, in a manner similar to well-known immunosuppressive factors, adenosine receptor (AR) agonists alter dendritic cell (DC) differentiation.

We used gene expression micro-array to gain information on the effects of AR ligation on monocyte-derived DC (mo-DC) differentiation and functions. Global transcriptional analysis suggested the effects of AR ligation operated largely autonomously from the differentiation process driven by cytokines. Furthermore, we identified several immunomodulatory factors (ILT-3, ILT-4 and CD25) and myeloid lineage differentiation markers (SCF, CD4 and CD123) differentially regulated at the protein level by AR agonists and by cAMP elevating agents. Collectively these changes, observed in immature DCs, were reminiscent of the phenotype associated with the so-called tolerogenic DC subsets. In addition this skewed differentiation resulted in an altered cytokine profile upon LPS maturation, the most notable feature being the near complete absence of secretion of the IL-12 family cytokines IL-12p70, IL-23 and IL-27. Lastly, from a functional point of view, these phenotypic changes translated into a defective capacity to prime antigen-specific CD8 T cells in vitro.

Our study thus offers insights into the developmental and immunomodulatory processes leading to the establishment of tolerogenic functions in DCs by adenosine as well as other factors using the cAMP second messenger pathway and suggests that such cues could contribute to the subversion of anti-tumor immunity by suppressing the initiation of CD8 T cell responses.

Key Words: CD8+ T cells, Dendritic cell.

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DENDRITIC CELLS AND TUMOR CELLS INTERNALIZE ALPHA-FETOPROTEIN, A HEPATOCELLULAR CARCINOMA TUMOR ANTIGEN, VIA DISTINCT ENDOCYTOSIS MECHANISMS

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Numerous cell surface receptors on dendritic cells (DC), including the mannose receptor (MR), DEC-205, DC-SIGN, and several scavenger receptors, mediate the endocytosis of exogenous antigen. Internalized antigen is then shuttled into lysosomes, where it is subsequently degraded and presented in the context of MHC Class

II molecules. Alternatively, DC are capable of re-routing internalized antigen into the MHC Class I pathway. This process, known as cross-presentation, is essential for the development of protective anti-tumor CD8+ T cell immunity. Importantly, recent evidence suggests that the divergence of internalized antigen into either MHC Class I or Class II pathways may be dictated by the particular cell surface receptor that was responsible for endocytosis of the antigen. We have recently shown that alpha-fetoprotein (AFP), an oncofetal antigen that is over-expressed by more than half of hepatocellular carcinoma (HCC) tumors, is efficiently internalized by human monocyte-derived DC. Furthermore, DC loaded with full-length AFP protein can stimulate both CD4+ and CD8+ antigen-specific T cell responses, indicating that AFP is being routed into both MHC Class I and Class II pathways. To identify the endocytosis mechanism(s) responsible for AFP internalization, we co-cultured DC with fluorescently-labeled AFP in the presence or absence of inhibitors of specific endocytic pathways. Pretreatment of DC with mannan, an MR inhibitor that competitively blocks endocytosis of mannose-rich structures, completely abrogated AFP uptake, while similar treatment with polyinosinic acid, a specific inhibitor of scavenger receptors, blocked over 80% of AFP internalization. These data are consistent with the observed high levels of MR and the scavenger receptors SR-A1 and CD36 expressed by DC. Because AFP is also taken up by HCC cells, where it is thought to regulate apoptosis and steroid receptor-mediated cell growth, we performed similar endocytosis blockade experiments using the HCC cell line HepG2. In contrast to DC, uptake of AFP by HepG2 cells, which express negligible levels of MR, was unaltered in the presence of mannan, while pretreatment of cells with polyinosinic acid yielded minimal yet reproducible inhibition of AFP uptake. We are currently testing the role of these distinct endocytosis mechanisms on the intracellular routing and MHC loading of internalized AFP. Collectively these data will enable the rational design of a DC-based vaccine for the treatment of HCC patients.

Key Words: Cancer vaccine, Dendritic cell.

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EXAMINING FACTORS LEADING TO PRODUCTION OF HIGHLY IMMUNOGENIC MONOCYTE DERIVED DCS SUITABLE FOR CANCER IMMUNOTHERPY IN COLOMBIAN INDIVIDUALS

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Vaccination with monocyte derived DCs pulsed with Tumor Associated Antigens HLA-A2 restricted (TAAs-A2) has been widely used for immunotherapy of tumors with variable clinical responses. It is possible that variable clinical response may be attributed to (i) generation of different quality of DCs depending upon the protocol used and/or to (ii) intrinsic functional differences of monocytes among individuals to respond either to differentiating cytokines or to maturation stimuli. CD80, CD86, HLA-DR, CCR7, CD83 and DC-SIGN expression in monocytes of 29 healthy Colombian volunteers was used to monitor the efficacy of IL-6, IL-1β, TNF-α and PGE-2 cytokines to differentiate into immune-competent DCs preparations of fresh vs. frozen- monocytes purified either untouched by negative selection or by plastic adherence and maintained in culture for two (Fast-DCs) or seven days (Standard-DCs) in GM-CSF/IL-4. Assays were conducted to determine the stimulation capacity of antigen-specific CD4+ and TAAs-A2-specific CD8+ T cells primed in vitro with peptide pulsed Fast-DCs or Standard-DCs.

In response to the maturation stimuli surface expression of CD86, HLA-DR and CCR7 were similar on immature (iDCs) and mature DCs (mDCs), in contrast, a remarkably over-expression of CD80, CD83 and marked down regulation of DC-SIGN in response to maturation was observed in mDCs suggesting these markers as reliable indicators to monitor DCs maturation. Despite a wide range of expression level of DC-SIGN was observed among individuals (ranged from 3 to 76% in iDCs) - upon maturation - a higher down-modulation of DC-SIGN was observed in Fast-DCs than in Standard-DCs. Notably the response of iDC to maturation stimuli assessed by these markers was not affected neither by the source of monocytes (fresh vs frozen) nor the isolation method (untouched vs adherence) or time of monocytes in culture (2d vs 7d). Functional assays with T cells showed that CFSE-labeled CD4+ T cells were efficiently stimulated either with mature Fast-DCs or Standard-DCs pulsed with tetanus toxoid, PHA and during mixed leukocyte reaction, and that the in vitro culture of CD8+ T cells from HLA-A2 individuals with peptide pulsed Fast-DCs evidenced HLA-A2 tetramer positive cytotoxic CD8+ T cells producing IFN-γ; IL-2 and TNF-α in response to NY-ESO1, Melan-A and HER-2 HLA-A2 restricted peptides. Our results suggest that Fast-DCs obtained from frozen monocytes by adherence lead to mDCs suitable for cancer immunotherapy in Colombian individuals.

Key Words: Adoptive therapy, Dendritic cell, Innate immunity.

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INNATE AND ADAPTIVE IMMUNE RESPONSE INDUCED EX VIVO BY AN IDIOTYPIC VACCINE FOR HCV-RELATED LYMPHOPROLIFERATIVE DISORDERS

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Hepatitis C virus (HCV) has been implicated as one of the major risk factors for type II mixed cryoglobulinemia (MC), an autoimmune disease that may evolve into an overt B cell non-Hodgkin's lymphoma (NHL) in about 10% of MC patients. It has been previously demonstrated that B cell receptor (BCR) repertoire, expressed by clonal B cells involved in HCV-associated type II MC as well as NHLs is not random, with the VK3-20 light

chain idiotype being one of the most represented, hence, selected as a target for passive as well as active immunization strategy.

Our preliminary studies showed that stimulation of antigen presenting cells with the VK3-20 protein induced the expression of activation/maturation markers such as CD83, CD86 and HLA-DR as well as the production of Th2 cytokines.

Aim of the present study was to test the effect on PBMC derived DCs and monocytes isolated from healthy donors and HCV+/MC+ patients. PBMCs were pulsed with 15µg/ml VK3-20 protein or VK3-20 conjugated with keyhole limpet hemocyanin (VK3-20-KLH) and stained for phenotypic analysis by flow cytometry. Moreover, production of cytokines was investigated in cell culture supernatants.

Cells pulsed with VK3-20-KLH showed a higher expression level of activation/maturation markers (i.e. CD40, CD80, CD83, CD86, HLADR) compared to those pulsed with non-adjuvanted VK3-20. Furthermore, a greater production of Th2 specific cytokines, including IL6, IL10, TNF alpha was observed in stimulated cells. In parallel, gene transcriptional profile of pulsed cells has been performed by microarray. Results from analysis of pathways as well as individual genes involved in the VK3-20 activation, with special focus on innate and early adaptive immunity, are currently in progress and will be discussed.

The overall results give an insight into mechanisms underlying the immune response induced by VK3-20 protein stimulation, to optimize its efficacy as idiotype vaccine approach.

Key Words: Cancer vaccine, Dendritic cell, Lymphoma.

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DC-NILV: A DENDRITIC CELL-TARGETING NON-INTEGRATING LENTIVIRAL VECTOR PLATFORM FOR DIRECT ADMINISTRATION IN IMMUNOTHERAPEUTIC CLINICAL SETTINGS

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Dendritic cells (DCs) are essential antigen presenting cells for the initiation and control of immune responses, therefore vaccines that deliver designated antigens directly to DCs in vivo are conceptually attractive. Immune Design Corp (IDC) is developing a vaccine platform around a novel class of non-integrating lentivectors that target DCs for the purpose of creating licensed immunotherapeutics. IDC's vector platform, termed DC-NILV (Dendritic Cell targeting Non-Integrating Lentiviral Vector), achieves DC targeting by utilizing a modified Sindbis virus envelope glycoprotein, SinVar1, which allows the transduction of DC via the DC-SIGN receptor. Transduction of human DCs was highly efficient using DC-NILV produced in the presence of mannosidase I inhibitors which promote the generation of SinVar1 glycoproteins with terminal mannose residues. Integration-defective lentiviral vectors will facilitate regulatory approval for clinical evaluation of vaccine candidates given by direct administration. We utilized a redundant approach to render DC-NILV integration defective by combining a pol gene encoding a mutant Integrase (D64V) with a self-inactivating (SIN) vector backbone deleted in the 3' LTR U3 region to the att site, and the 3' LTR-proximal polypurine tract. This composition favors formation of single-LTR reverse transcribed episomal dsDNA circles in infected DCs, which are not a template for chromosomal integration. Unprocessed titers of DC-NILV produced by transient transfection are $>5 \times 10^6$ infectious units (IU)/mL and we have developed chromatography-based methods for large scale ($>3 \times 10^{11}$ IU) production, together with appropriate release specification for clinical evaluation. Prime and prime-boost immunization regimens with 1×10^7 IU of DC-NILV encoding model antigens, such as LCMV gp33 and OVA, induced polyfunctional primary and secondary CD8 T cell immunity. A single immunization with 1 x 107 IU of DC-NILV encoding OVA induced a stable CD8 T cell memory population of ~2% that expanded more than 5-fold and provided 4 logs of protective immunity against challenge 1 month post-immunization with 1×10^7 PFU of vaccinia virus encoding OVA. DC-NILV encoding AH1-A5, an endogenous rejection Ag for CT26 tumor cells, induced ~4% primary CD8 T cell immunity, and a single injection conferred 70% long-term survival in BALB/c mice bearing CT26 tumors. Based on these results and methods for efficient large-scale production and purification, we are developing a DC-NILV vaccine candidate for a first-in-human evaluation in the setting of cancer.

Key Words: Cancer vaccine, CD8+ T cells, Dendritic cell.

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HUMAN LANGERHANS DENDRITIC CELLS STIMULATE ROBUST CYTOLYTIC T-CELLS AGAINST TUMOR ANTIGENS, INCLUDING WT1, BY AN IL15-DEPENDENT MECHANISM

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Human Langerhans dendritic cells (LCs) are the most potent conventional DC subtype for stimulating CTLs against viruses and tumors in vitro. Compared with other conventional human DC subtypes, LCs produce the most IL-15. LCs also synthesize 3-5 fold higher amounts of IL15Rα than do moDCs ($P<0.001$ to 0.05), based on measurement of mRNA by RT-PCR or detection of receptor protein by digitalized immunofluorescent imaging. Freshly isolated LC crawlouts from human epidermal sheets also express IL15Rα. LC-stimulated T cells strongly upregulate pSTAT5, an early T cell activation event, with no enhancement by exogenous IL15 (10 ng/ml) (P=NS). In contrast, moDCs need exogenous IL15 to induce a comparable pSTAT5 response by T cells ($P= 0.008$). IL15Rα blockade of either moDCs or LCs completely inhibits pSTAT5. LCs thus have abundant IL15Rα to shepherd IL15 to the cell surface for presentation in trans

to responder lymphocytes expressing IL15R-βγ. To capitalize on a broadly expressed, self-differentiation tumor antigen, LCs generated from CD34+ progenitors from healthy individuals underwent electroporation with WT1-mRNA. These LCs stimulate robust WT1-specific autologous CTLs, after a single 7d round of stimulation at a T cell:LC ratio of only 10 or 30:1, in the absence of exogenous IL-15. These CTLs lyse 85-95% of targets from a WT1+ tumor cell line and 63% (+/- 11% SEM) of primary WT1+ blasts from patients with AML in an HLA-restricted manner. MoDCs are completely incapable of generating WT1-specific CTLs under comparable conditions and require exogenous IL-15 to generate 70-80% specific lysis of WT1+ targets. Anti-IL15Rα opsonization of LCs during their 7d priming of T cells abrogates their ability to stimulate CTLs (P = 0.001). Activation of T cells by LCs therefore results in stimulation of both antigen recognition and IL-15 receptor pathways, leading to a fundamentally different set of intracellular signals from those arising during antigen presentation by moDCs. These data connect the abundant expression of the IL15/IL15Rα complex by human LCs to their superior stimulation of potent anti-tumor CTLs, which moDCs cannot achieve without exogenous IL15. These results support the use of mRNA-electroporated LCs as vaccines for cancer immunotherapy, thus overcoming tolerance against self-differentiation antigens shared by tumors.

Key Words: Active immunotherapy, Cancer vaccine.

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P53 DENDRITIC CELL VACCINATION IN PATIENTS WITH HEAD AND NECK SQUAMOUS CELL CANCER

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Background. Patients with advanced head and neck squamous cell cancer (HNSCC) have a poor prognosis, and tumor specific vaccination has been proposed as a means to improve therapeutic efficacy. The tumor suppressor, p53, is the most frequently altered gene in HNSCC, providing a useful immunotherapeutic target antigen.

Methods. HLA-A2.1+ HNSCC patients with no active disease were vaccinated with autologous, monocyte-derived dendritic cells (DC) pulsed with two wild type (wt) p53 peptides (Arm 1) +/- T helper tetanus toxoid peptide (Arm 2) or +/- Th wt p53 peptide (Arm 3). Conventionally matured DC were phenotyped for surface markers (CD80, CD83, CD86, HLA-DR, CCR7) by flow cytometry, and cytokine production (IL-10, IL-12) was measured by ELISA. Expression of antigen-processing machinery (APM) components TAP1/2, calnexin and calreticulin were measured by flow cytometry. Immune monitoring at baseline, on days 28 and 49 after conclusion of the vaccination included whole blood counts, the frequency of vaccine-specific T cells by tetramer analysis, polyfunctional cytokine expression in T cells by ten-color flow cytometry, the frequencies of CD4+CD39+CD25+ regulatory T cell (Treg) and CD4+CD39+CD25neg ex-Treg populations in the peripheral blood.

Results. Vaccinations were well tolerated by HNSCC patients. In vitro matured DC up-regulated surface costimulatory/adhesion markers, but not TAP1 (MFI: 0.3 ± 0.2) or TAP2 (MFI: 0.4 ± 0.2). Patients with increased p53 tetramer+ T cells (8/16) in the periphery after vaccination generated DC which produced more IL-12 (15 ± 14 pg/mL) and less IL-10 (140 ± 159 pg/mL) than patients without tetramer response (IL-12: 6 ± 6 pg/mL, IL-10: 175 ± 197 pg/mL). In polyfunctional cytokine analyses, Th1 cytokine expression upon T-cell receptor stimulation was significantly increased in 69% (11/16), while frequencies of Treg and exTreg were decreased in 88% (14/16) in patients after vaccination (p = 0.006). All patients with a tetramer response remained NED at last follow up (>24 mo).

Conclusion. Vaccination of HNSCC patients with wt p53 peptide loaded DC is feasible and safe. However, HNSCC patients' DC respond poorly to standard maturation stimuli. Decrease of Treg frequency and increase of Th1 cytokine expression will need to be enhanced by additional maturation stimuli to maximize clinical response.

Key Words: Cancer vaccine, Dendritic cell, Phase I.

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A PILOT TRIAL OF WT1 PEPTIDE-LOADED ALLOGENEIC DENDRITIC CELL (DC) VACCINATION AND DONOR LYMPHOCYTE INFUSION (DLI) FOR WT1-EXPRESSING HEMATOLOGIC MALIGNANCIES AND POST-TRANSPLANT RELAPSE

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Background: Treatment of relapse after allogeneic hematopoietic stem cell transplantation (AlloHSCT) remains challenging. The Wilms' tumor 1 (WT1) gene product is a tumor-associated antigen expressed in many hematologic malignancies. This trial incorporates antigen-specific immunotherapy and allogeneic adoptive cell transfer for pediatric and adult patients with relapsed hematologic malignancies after AlloHSCT. The primary aims are to assess safety and feasibility of a novel vaccine designed to enhance the graft-vs-leukemia effect.

Design: HLA-A2+ patients with WT1-expressing hematologic malignancies that have relapsed after AlloHSCT are eligible. Donor-derived DC vaccines are given every 2 weeks for 6 doses and DLI every 4 weeks for 3 doses. Peripheral blood monocyte-derived

DCs are loaded with a combination of three HLA-A2 binding WT1 peptides. In attempt to enhance antigen presentation, peptides include an 11-mer TAT protein transduction domain. Study endpoints include toxicity, feasibility, antigen-specific immune response, and clinical response.

Results: 4 patients, aged 9-19 years were treated, 3 with acute lymphoblastic leukemia (ALL) and one with Hodgkin lymphoma. Vaccines were successfully produced. All patients tolerated vaccine and DLI administration well. The most common adverse events were mild, reversible pain and pruritis at vaccine sites and delayed type hypersensitivity (DTH) skin test sites. One patient developed Grade I skin GVHD not requiring systemic therapy. All 3 patients with ALL demonstrated positive ELISPOT responses to WT1-peptides and positive DTH responses to the KLH control. 2/3 ALL patients showed DTH responses to the WT1-peptides. No immune response was observed in the patient with Hodgkin lymphoma. 1 patient with ALL remains in remission 10 months after initiation of therapy and 3 have died of disease. Median overall survival was 12 months.

Conclusions: This novel allogeneic immunotherapy regimen is feasible, well tolerated and can induce tumor directed immune responses in patients with relapsed ALL following allogeneic stem cell transplant. Accrual is ongoing.

Key Words: Adoptive therapy, Dendritic cell, Leukemia.

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HIGH-THROUGHPUT ANALYSIS OF PLASMACYTOID DENDRITIC CELLS REVEALS DIFFERENTIAL RESPONSES TO DISTINCT VIRAL PATHOGENS

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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, protein, and cellular 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. 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 high dose H1N1 can stimulate both pDC and T cell 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.

Key Words: Dendritic cell, HIV.

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THE HOST STING PATHWAY IS CRITICAL FOR INNATE IMMUNE SENSING OF A GROWING TUMOR AND BRIDGING TO AN ADAPTIVE IMMUNE RESPONSE VIA IFN-β

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Adaptive T cell responses are required for effective anti-tumor activity, and spontaneous T cell responses against tumors occur frequently. However, the mechanisms by which innate immune responses become induced in response to cancer, and how they can bridge to T cell priming against tumor antigens, are poorly defined. We recently showed that CD11c+ cells produce IFN-β after tumor implantation and this IFN-β plays a critical role on intratumoral accumulation of CD8α+ dendritic cells. As such, spontaneous tumor antigen-specific T cell priming was defective in IFNα/βR or Stat1 knockout mice, and also in Batf3-deficient mice which lack the CD8α+ DC subset. Based on these results, it has become critical to identify the sensing mechanism that mediates production of IFN-β by host DCs in response to tumor-derived products. Using specific gene targeted mice, we observed that expression of MyD88, TRIF, or P2X7R was not required in host APCs for spontaneous T cell priming. In addition, no evidence was obtained to support a role for the inflammasome pathway in type I IFN production. In contrast, mice deficient in the molecule

STING (stimulator of IFN gene) were severely deficient in IFN-β production and T cell priming against tumors. In models of spontaneous tumor rejection, tumors grew progressively in STING-/- mice. Bone marrow derived dendritic cells from STING-/- mice showed markedly defective IFN-β production in vitro. Gene expression profiling of stimulated STING-/- DCs revealed defective upregulation of numerous additional factors, including CD40, CD86, CXCL9, IL-6, and TNF, suggesting a critical role for this pathway in a broad array of DC activation parameters. Our data

suggest that the STING pathway in innate immune cells is one of the central mechanisms for IFN-β production and DC activation in response to tumor recognition and is required for effective spontaneous priming of anti-tumor T cells in vivo. Manipulation of this pathway could have important therapeutic implications.

Key Words: Cytokine, Dendritic cell, Innate immunity.

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POLYMERIC NY-ESO-1 ENGAGES IMMATURE DENDRITIC CELLS THROUGH CELL-SURFACE RECEPTORS: A TUMOR-ASSOCIATED ANTIGEN AS WELL AS A DAMAGE-ASSOCIATED MOLECULAR PATTERN

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In search of intrinsic factors that contribute to the distinctively strong immunogenicity of a non-mutated cancer/testis antigen, we found that NY-ESO-1 forms polymeric structures through disulfide bonds. NY-ESO-1 binding to immature dendritic cells (DC) was dependent on its polymeric structure and involved toll-like receptor-4 (TLR4) in addition to complement c1q receptor (CRT) on the surface of immature DC in mouse and human. Gene gun-delivered plasmid encoding the wild-type NY-ESO-1 readily induced T cell-dependent antibody (Ab) responses in wild-type C57BL/10 mice but not TLR4-knockout C57BL/10ScNJ mice. Disrupting polymeric structures of NY-ESO-1 by cysteine-to-serine substitutions lead to diminished immunogenicity and altered TLR4-dependence in the induced Ab response. To demonstrate its adjuvant effect, NY-ESO-1 was fused with a major mugwort pollen allergen Art v 1 and a tumor-associated antigens (TAA) carbonic anhydrase 9. Plasmid DNA vaccines encoding the fusion genes generated robust immune responses against otherwise non-immunogenic targets in mice. Polymeric structure and TLR4 may play important roles in rendering NY-ESO-1 immunogenic and thus serve as a potential molecular adjuvant. This study provides a unique example of a TAA that is also a damage-associated molecular pattern (DAMP).

Key Words: Adjuvant, Dendritic cell, Tumor associated antigen.

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12-COLOR MULTI-PARAMETER FLOW ANALYSIS OF DENDRITIC CELL SUBSETS IN HUMAN TUMORS AND ADJACENT LYMPH NODES

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In recent work we described 4-color flow-based phenotypic, as well as, functional characterization of four conventional dendritic cell (cDC) subsets within human tumor negative, skin-draining sentinel lymph nodes (LN) from early-stage melanoma patients. In the current study, tumor digests at various stages, as well as adjacent (and sentinel) LNs were interrogated for the activation status of different DC subsets (both cDC and pDC), as well as for frequencies of MDSC, T- and B cells. Making use of 12-color polychromatic flow cytometry, it was evident that cDC activation, based on CD80, CD86, CD83, HLA-DR and CD25 expression, was drastically reduced with increased tumor burden (tumor negative LN vs. tumor positive LN vs. tumor digests). In LNs and tumor digests of two advanced-stage melanoma patients, the C-type lectin CLEC9A, which has been described to be present on human CD11c+ BDCA3+ blood and splenic DC and identifies DCs with enhanced cross-presenting abilities, was found to be expressed in low frequencies on CD3+ cells, CD19+ HLA-DR+ cells and a subset of, as yet unidentified, (CD3- CD19-) CD11c- cells. No CD11c+ BDCA3+ CLEC9A+ cells have been observed in LNs or tumor samples thus far, indicating such a population, if present at all, to be extremely rare. In addition the analysis revealed the presence of CD80hi CD86- HLA-DRlow CD83- cells, which expressed the inhibitory ligand B7H4, as a sub-population amongst the otherwise mature skin-derived CD11c_hi CD1a+ dermal DC and the CD11c_int CD1a+ Langerhans cells within the LNs. Further functional analysis will be required to reveal whether these cells, compared to CD80+/- CD86+ HLA-DRhi CD83+ cells (expressing B7H4 and/or PD-L1) harbor immune-suppressive effects. Overall we aim to build a comprehensive data set, which will shed light on the different DC subsets present in human LNs and corresponding tumors, their activation status and their functions.

Key Words: Dendritic cell, lymph node, flow cytometry.

Presenting author underlined. *Priminary author in italics.*

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MYELOID-DERIVED SUPPRESSOR CELLS IN MELANOMA PATIENTS: GENESIS AND ROLE IN TUMOR-MEDIATED IMMUNE SUPPRESSION

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Tumor immune tolerance is associated with the recruitment, differentiation and expansion of suppressor cell populations, including myeloid-derived suppressor cells (MDCS). Thanks to their pro-angiogenic activity and the ability to blunt tumor immunity, these cells are supposed to play a central role in local and systemic tumor progression. We recently reported that melanoma patients with advanced disease, poorly responding to cancer vaccine administration, have in their peripheral blood a specific and consistent accumulation of immunosuppressive CD14+CD11b+HLA-DRneg/low monocytes inhibiting T cell immunity though TGFβ. Interestingly, these cells can be also detected in PBMC of patients with initial disease and even in primary lesions as infiltrates surrounding tumor nests, suggesting an early involvement of this suppressive pathway in melanoma. Cells with comparable phenotypic and functional features can be also generated by culturing monocytes from healthy donors with exosomes isolated from melanoma cell supernatants. This in vitro model, beside having a possible in vivo counterpart, represents an optimal setting for understanding the mechanisms involved in MDSC-mediated immune suppression and possibly developing specific therapeutic strategies selectively targeting these pathways. We recently extended the analysis by immunoprofiling monocytes incubated with melanoma exosomes. As detected by CBA, Multiplex technology platform and RT-PCR, exosome-treated monocytes release a defined array of soluble factors (including IL-6, IL-8, IL1β and VEGF), that could play a role in promoting MDSC expansion and tumor progression. Interestingly, melanoma-derived exosomes have also a rich immune-related content, as they express high levels of soluble factors (such as CXCL1, VEGF-A, TGFβ-1) and related mRNA. Of note is the evidence that the immune signatures detected in melanoma exosomes seems to reflect those detected in exosome-treated monocytes, suggesting the ability of these nanovesicles to directly and specifically transferring immunosuppressive properties to recipient cells. These data point to exosomes as a possible tool used by tumor cells to mould host microenvironment and favour cancer progression. Interfering with this process may thus represent a novel strategy for retrieving effective immune control in cancer patients.

In conclusion, immunosuppressive pathways interfering with tumor immunity in cancer patients represent a key area still needing to be dissected in terms of molecular mechanisms, clinical relevance, and suitability as novel target for innovative cancer therapies.

Key Words: Melanoma, Myeloid derived suppressor cell, Tumor microenvironment.

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TARGETING REGULATION OF INFLAMMATION IN COLON CANCER

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The role of Treg in colon cancer is poorly understood. Treg are well known for their ability to suppress tumor specific cytotoxic T-cells (Khazaie et al., 2009; Khazaie and von Boehmer, 2006). Surprisingly, recent reports suggest that tumor infiltration by Treg is a favorable prognostic factor in CRC (Correale et al., 2010; Salama et al., 2009). These observations are compatible with our studies in mice and have to do with the ability of Treg to suppress cancer inflammation (Gounaris et al., 2009; Gounaris et al., 2007; Gounaris et al., 2008; Khazaie et al., 2011). However, we recently reported, for the first time, gain of pro-inflammatory properties by both tumor infiltrating and circulating Treg in colon cancer patients (Blatner et al., 2010) and in mice with polyposis (Colombo and Piconese, 2009; Gounaris et al., 2009). This raises questions about protective versus pathogenic Treg, and how to distinguish the two. Functional heterogeneity of Treg is now extensively discussed (Bailey-Bucktrout and Bluestone, 2011; Feuerer et al., 2009; Hori, 2011), and tumor-promoting properties of Treg is attracting attention (Tan et al., 2011). Our observations are compatible with the existence of both pathogenic and protective Treg subsets among tumor infiltrating cells and in circulation in colon cancer. We provide evidence for a distinct subset of RORγt expressing Treg with pathogenic pro-inflammatory properties that expand in colon cancer in a tumor dependent manner and critically contributes to disease outcome. Targeting these pathogenic Treg tip the balance in favor of protective immunity while avoiding autoimmunity.

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

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A KEY WAS FOUND NEAR THE LOCK: IMMUNOGLOBULIN REPERTOIRE ANALYSIS OF TUMOR INFILTRATING B LYMPHOCYTES IN MELANOMAS REVEALED OVERREPRESENTED IMMUNOGLOBULIN VARIABLE REGIONS SPECIFIC FOR HIGHLY TUMOR ASSOCIATED ANTIGENS

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Background and Objectives: Only very limited knowledge has gathered about the B cells infiltrating solid tumors (Tumor infiltrating B lymphocyte, TIL-B) especially melanomas. Their small quantities hindered the special investigations. There was a need for novel approaches to boost the understanding of the mechanisms underlying natural antibodies' responses to tumor antigens. Our project aims to define the potential capacities of B cells present in malignant melanomas. Methods: Thirty superficial spreading melanoma (SSM) and nodular melanoma (NM) tissue samples were selected to make tissue microarrays (TMA) (TMA Master for MIRAX Viewer 1.12, 3D Histech) for immunohistochemistry with specific B cell markers and different melanoma tumorassociated antigens. Immunoglobulin rearranged variable region VH - JH and VK - JK genes in 6 selected cases were amplified by RT-PCR, cloned and sequenced. Results: Comparative DNA sequence analysis of 218 cloned heavy and light chain immunoglobulin variable region genes were succesfully performed with Vector NTI Advance 11 (Invitrogen). Single chain Fv (scFv) antibody fragment libraries were sucessfully constructed and tumor binder antibody fragment clones could be selected, when tested against tumor membrane preparations of various melanoma cell lines (SK Mel 28, M1/9, HT999, HT168. Our data analysis revealed immunoglobulin variable region genes with highly tumor associated sialilated glycosphingolipide antigen, ganglioside binding potential among the overrrepresented clones.

Conclusions: Immunohistochemical studies revealed the special B cell phenotype of these TIL-Bs in melanomas. The comparative DNA analysis revealed the main characteristics of TIL-B immunoglobulin repertoire with overrepresented families and clusters with oligoclonal predominance. Our data indicate internationally as first that tumor associated sialilated glycosphingolipides, GD3 gangliosides are among the target molecules of these TIL-B immunglobulins, strongly expressed on melanomas. We postulate that the analysis of the antibody repertoire of these TIL-B cells could lead to the further understanding of the precise nature of natural antibodies to tumor antigens.

Acknowledgements: We acknowledge the Award of the Harry J. Lloyd Charitable Trust

Key Words: B cell, Melanoma, Tumor associated antigen.

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HIGHER AVIDITY MESOTHELIN-SPECIFIC CD8+T CELL REPERTOIRES ARE ASSOCIATED WITH LONGER DISEASE-FREE SURVIVAL AND TUMOR-TRAFFICKING IN PATIENTS TREATED WITH A GM-CSF-SECRETING PANCREATIC TUMOR VACCINE

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Mesothelin is over-expressed by a variety of tumors and likely serves as a cell adhesion molecule conferring metastatic potential. We previously reported the induction of mesothelin-specific CD8+ T cell responses in patients treated with an allogeneic GM-CSF-secreting pancreatic tumor vaccine. Importantly, these responses correlated with both increased DTH responses to autologous tumor and prolonged disease-free survival (DFS). More recently, we evaluated CD8+ T cell responses to an expanded set of mesothelin peptides in patients treated on a larger phase II follow-up study. Collectively, these studies suggest that the maintenance and expansion of the mesothelin-specific T cell repertoire may distinguish clinical responders from non-responders. However, these measures cannot provide an early assessment. Furthermore, alternative immune measures may more accurately differentiate responders from non-responders.

The purpose of this study was to evaluate differences in mesothelin-specific T cell avidities between clinical responders and non-responders on the follow up study. T cell avidity was assessed by tetramer dilution analysis performed directly ex vivo on peripheral blood lymphocytes (PBL) isolated prior to and following the 1st vaccination and again following the final vaccination from HLA-A2+ patients. For each of the 6 mesothelin peptides evaluated, higher T cell avidity was associated with longer DFS. Importantly, this trend was observed at each of the timepoints tested, even at baseline. Furthermore, more diverse higher avidity mesothelin-specific T cell repertoires were associated with longer DFS. These data suggest that T cell avidity may provide an early marker for differentiating responders from non-responders. We have also performed tetramer dilution analyses on PBL from patients treated on an ongoing vaccine study. In this study, the 1st vaccine is given 2 weeks prior to surgery, providing the unique opportunity to

study post-vaccination intratumoral responses. Tetramer analysis of uncultured lymphocytes isolated from resected tumor tissue (TIL) demonstrated that mesothelin-specific T cells were only present in TIL isolated from patients bearing higher avidity peripheral T cell repertoires. Importantly, these data demonstrate for the 1st time that mesothelin-specific T cells are involved in the local anti-tumor response in some patients and may explain why peripheral responses have correlated with prolonged DFS. Additional studies are required to pinpoint which peripheral immune measures correlate best with tumor trafficking and improved DFS.

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

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USE OF FRESH WHOLE BLOOD TO IDENTIFY EXPANSIONS IN GRANULOCYTES AND MONOCYTES IN PATIENTS WITH PANCREATIC ADENOCARCINOMA

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Expansion of peripheral blood myeloid cells has been correlated with the presence of malignancy in patients. $CD11b^{+}Gr1^{hi}$ cells with granulocyte nuclear morphology make up the greatest proportion of expanded myeloid cells in the commonly studied 4T1 and 3LL murine transplantable tumor models. In cancer patients, conventional PBMC preparations and cryo-preservation leads to a loss of granulocyte populations, which are the largest proportion of myeloid cells in the peripheral blood. For these reasons, we developed a quantitative FACS assay to measure myeloid cell number and phenotype in fresh whole blood of patients with a diagnosed pancreatic mass who are candidates for resection. Recruitment: 56 patients who were scheduled to undergo pancreatic resection in our institution from May 2010 until July 2011 enrolled in the study and provided blood samples. 28 of these patients were found to have pancreatic adenocarcinoma. Results: While CD33 was a useful pan-myeloid marker, the combination of CD15, CD14, HLA-DR and side-scatter was superior in distinguishing $CD33^{+}CD15^{+}SSC^{hi}HLADR^{-}$ granulocytes from $CD33^{+}CD14^{+}SSC^{int}HLADR^{+/-}$ monocytes. The identity of each population was confirmed by FACS sort and morphologic analysis. Granulocyte expansions were only identified in patients with pancreatic adenocarcinoma, and not in patients found to have benign or pre-malignant disease. Moreover, among patients with pancreatic adenocarcinoma, the expansions were associated with invasive disease. Conclusions: Pancreatic adenocarcinoma has proven to be difficult to treat with conventional therapies and immunotherapy, which may be a consequence of its propensity to generate biological processes characteristic of wound repair: neoangiogenesis, fibrosis, and immunosuppression. We propose that these myeloid expansions in cancer patients are a result of normal responses to tissue damage as a result of invasive carcinoma. We propose that myeloid expansions in the peripheral blood may be useful to identify those patients with advanced malignancy who may be candidates for therapies that alter the phenotype of myeloid cells within the tumor environment.

Key Words: Innate immunity, Macrophages, Tumor microenvironment.

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FAILURE OF CD8+ T CELLS OF RENAL CELL CARCINOMA INFILTRATING LYMPHOCYTES TO MEDIATE GRANULE EXOCYTOSIS IS REGULATED BY HIGH DGK-α AND A BLUNTED MAP KINASE PATHWAY AND CAN BE REVERTED BY IL2

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Tumor-infiltrating CD8+ T cells are found in many types of tumors including human renal cell carcinoma (RCC) and when tested ex vivo they often are able to recognize and kill tumor cells. However, tumor rejection rarely occurs suggesting limited functional activity in the tumor microenvironment. Understanding the functional deficits leading to suppressed antitumor response will be a prerequisite to improve cancer immunotherapy. CD8+ T cells of TILs of RCC were found to be unresponsive to CD3 stimulation ex vivo showing no granule exocytosis and no lytic activity. CD8 T cell function requires TCR signal transmission via cascades of phosphorylation events of signaling molecules. Using phosphoflow cytometry and multiplex phosphoprotein array it was observed that phosphorylation levels of proximal signaling molecules LCK and PLC-γ were similar in CD8+ T cells of TILs compared to PBLs of healthy donors, while TCR distal MAP kinases, ERK and JNK, and AKT showed reduced phosphorylation levels after stimulation. These deficits were tumor-specific as they were not observed in normal kidney infiltrating CD8+ T cells (NILs). Diacylglycerol kinase alpha, which metabolizes diacylglycerol to phosphatidic acid and therefore affects downstream signaling molecules, was found to be higher expressed in CD8+ T cells of TILs compared to NILs. To test whether the deficits could be reverted two strategies were explored: inhibition of DGK-α and provision of IL2, which is known to support T effector cell function. DGK-α-inhibition during ex vivo CD3 stimulation improved lytic granule exocytosis of CD8+ T cells of TILs. Cultivation of TILs in low dose IL2 reduced the DGK-α protein level and increased the constitutive phosphorylation of ERK. Upon CD3 stimulation, IL2-cultured CD8+ T cells of TILs showed higher percentages of degranulating cells and the activation of ERK as well as of AKT was higher than in noncultured CD8+ T cells of TILs reaching phosphorylation levels comparable to those found in CD8+ T cells of noncultured NILs and PBLs. Additionally, the

protein level of the AKT client molecule p27kip, an inhibitory cell cycle protein, was reduced, while cyclin E, which promotes transition from G1 to S phase, was increased. These results indicate that the deficits of TILs are reversible. DGK-α-inhibition and provision of IL2 signals could be strategies to recruit the natural CD8+ T cells to the antitumor response and may help to prevent inactivation of adoptively transferred T cells thereby improving therapeutic efficacy.

Key Words: CD8+ T cells, Renal cell carcinoma, Tumor infiltration lymphocytes.

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MYELOID DERIVED SUPPRESSOR CELLS FUNCTION AS OSTEOCLAST PROGENITORS ENHANCING BONE DESTRUCTION IN BREAST CANCER

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Myeloid derived suppressor cells (MDSC) numbers are elevated with increased tumor grades. MDSC inhibit the anti-tumor immunity and promote tumor expansion and metastasis including to bone. Bone is the predominant metastatic site for osteolytic cancers. Osteolytic bone metastasis is associated with increased morbidity and mortality in breast cancer (BCa) patients with median survival rate of less than 2 years. As cancer cells metastasize to bone, they induce osteoclast activation, which degrades bone thus allowing cancer growth. As MDSC are the progenitors of macrophages, which differentiate into osteoclasts, and their numbers are elevated in BCa patients, we hypothesized that MDSC may differentiate into osteoclasts and contribute to enhanced bone destruction and tumor growth.

Towards this goal, BALB/c mice were injected with 4T1 BCa cells via intra-cardiac route. Once bone metastasis was established, CD11b+Gr-1+ MDSC population was isolated from bone marrow. MDSC were then cultured in vitro in the presence of M-CSF and RANKL. As a positive control, bone marrow derived macrophages were included in all assays. Formation of multi-nucleated tartarate resistant acid phosphatase (TRAP)-positive osteoclasts were apparent in MDSC cultures. Functional property of this osteoclast like cells was confirmed as they formed pits on the bone slices. Further, it was observed that as MDSC differentiated into osteoclasts they expressed various osteoclast specific markers such as carbonic anhydrase 2, cathepsin K, MMP-9 and TRAP. MDSC are known to exert the immunosuppressive effect with increased nitric oxide (NO), reactive oxygen species (ROS) production and arginase expression. Therefore, we examined if any of these mechanisms contribute to differentiation of MDSC into osteoclasts. Results of this study clearly showed that as MDSC differentiated into osteoclasts, nitric oxide production was significantly increased and addition of a specific nitric oxide synthase inhibitor completely blocked osteoclast differentiation.

Collectively, the present study reports a novel role for MDSC as osteoclast forming cells, which contribute to bone destruction during BCa bone dissemination. As MDSC are elevated in other osteolytic cancers, it is speculated that such osteolytic potential of MDSC may play a vital role in increased bone destruction and growth of tumors in the bone microenvironment. Studies so far from the ER+ BCa patients indicated the presence of CD11b+HLA-DR-CD33+CD14+CD15- and CD11b+HLA-DR-CD33+CD14-CD15+ MDSC populations. Results of ongoing studies to determine if these populations of MDSC differentiate into osteoclasts will be further discussed.

Key Words: Breast cancer, MDSC, Osteoclasts.

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LONG TERM SURVIVAL IN GLIAL BRAIN TUMORS OF CHILDREN AND ADULTS CORRELATES WITH IMMUNE RESPONSE IN THE TUMOR

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To identify novel prognostic markers for glial brain tumors, my laboratory has employed unbiased genome-wide microarray analysis of archived glioblastoma(GBM) and ependymoma (EPN) tumor specimens from adults and children with known clinical outcomes. Gene expression microarray data were combined with computer-based gene ontology analyses Gene Set Enrichment Analysis (GSEA) and Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify sets of functionally related genes that were associated with a good clinical outcome. Unexpectedly, this approach revealed that a significant overexpression of immune genes is the predominant feature that distinguishes long term survival in the two glial tumor types studied. Immune genes associated with a survival in GBM were strongly indicative of a T-cell response, whereas those associated with survival in EPN were predominantly microglia-associated genes. It was hypothesized that the expression of immune genes identified by microarray analysis were restricted to tumor-infiltrating immune cells. This was shown to be the case by histological examination of outcome associated immune genes AIF1 and HLA-DR. It was also found that increased tumor infiltration by CD4+ and CD8+ T-cells and microglia distinguished long term from short term survivors of both GBM and EPN. Based on these data, we hypothesize that the host immune system is critically involved in controlling tumor growth in long-term survivors of GBM and EPN. These findings provide a novel prognostic tool and strong rationale for the use of immunotherapy against brain tumors.

Key Words: ependymoma, glioblastoma, Infiltrate.

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GM-CSF EXPRESSION IS ASSOCIATED WITH IMPROVED SURVIVAL IN COLORECTAL CANCER

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Background: In previous studies based on the staining of two tumor microarrays (TMA) collectively including over 1600 annotated specimens we found that infiltration of colorectal cancer (CRC) tissues by CD16+ myeloid cells is associated with significantly improved prognosis (Sconocchia et al., Int J Cancer. 2011;128:2663-2672). Based on this background in this work we addressed mechanisms potentially underlying this beneficial effect.

Methods: Peripheral blood CD14+ cells from healthy donors were stimulated for six days in the presence of GM-CSF or M-CSF and co-cultured for three days with Colo 205 CRC cells. 3H-Thymidine incorporation was then used to assess tumor cell proliferation. Total cellular RNA was extracted from CRC and corresponding autologous healthy mucosa (HM) specimens, reverse transcribed and amplified in the presence of primers and probes specific for genes encoding different cytokines (CK) in quantitative, real-time PCR (qRT-PCR). A TMA including >1200 annotated CRC specimens was stained with a GM-CSF specific antibody and correlations of positive staining with clinico-pathological features and overall survival were evaluated.

Results: Culture in the presence of GM-CSF at 25-6.2 ng/ml concentrations induced CD16 expression in 65±6% of monocytes, as compared to 22±5% in cells cultured in the presence of M-CSF ($p=0.0041$). Following stimulation with GM-CSF but not M-CSF these macrophages were able to inhibit Colo 205 CRC cell proliferation by 65-35% ($p<0.02$) depending on CK concentrations used in the activation. Cytostatic effect required cell contact and was not accompanied by induction of apoptosis in CRC cells. Based on these data, we analyzed CK gene expression in CRC and autologous healthy mucosa. We found that GM-CSF gene is expressed to significantly ($n=45$, $p<0.001$) higher extents in CRC than in HM whereas M-CSF gene expression is higher in HM than in CRC ($p<0.05$). In keeping with these data, genes encoding typical M1 genes, including IL-1β, TNFα, iNOS and IL-23 are expressed to significantly ($p<0.005$) higher extents in CRC than in HM. TMA staining with anti GM-CSF, revealed that lower specific staining is associated with larger tumors ($p=0.027$), an infiltrating growth pattern ($p=0.004$) and a significantly ($p=0.006$) shorter survival in mismatch-repair proficient (MMR-P) CRC cases.

Conclusions: Our data suggest that GM-CSF might favourably influence CRC clinical course by promoting M1 macrophage differentiation.

Key Words: Colorectal cancer, Cytokine, Tumor microenvironment.

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SPECIFIC RECOGNITION OF METASTATIC GASTROINTESTINAL CANCERS BY TUMOR INFILTRATING LYMPHOCYTES

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Tumor infiltrating lymphocytes (TIL) are described in all solid tumors, but have convincingly been shown to recognize autologous tumors only in melanoma. Here we investigated whether TIL expanded from metastatic gastrointestinal (GI) carcinomas recognize autologous cancer cell lines with specificity.

Methods: Cell suspensions from fresh GI cancer metastases and peripheral blood lymphocytes (PBL) from the same patients were assessed by flow cytometry. Cancer cell lines and TIL were grown from metastatic deposits. CD8+ TIL reactivity was assessed by interferon-gamma (IFN-g) release in the supernatant and cell surface marker expression by flow cytometry after 24 hrs coculture assays. Tumor lysis was assessed by Cr51 release assays. TIL clones were obtained by limiting dilution after enrichment of 4-1BB positive TIL post coculture. TCRs from TIL clones were sequenced after 5'RACE of total RNA extracts and TA cloning.

Results: In seven patients with GI cancers, lung and liver metastases harbored significantly more 4-1BB and PD-1 positive effector memory CD8+ T cells than present in the autologous PBL ($2.0 \pm 0.3\%$ versus $0.2 \pm 0.1\%$ on average for 4-1BB positive cells, and $22.9 \pm 4.1\%$ versus $1.1 \pm 0.4\%$ for PD-1; $p<0.01$ for both comparisons). New cancer cell lines could be established for two tumors. In a first patient with gastric cancer, the bulk population of CD8+ TIL from liver metastases was reactive to autologous cancer cell lines with high specificity. Nineteen reactive CD8+ TIL clones out of 154 tested were reactive by upregulation of 4-1BB expression. Six clones secreted significant amounts of IFN-g in contrast with seven others, which markedly up-regulated PD-1. All TIL clones lysed the autologous cancer lines with specificity, but the high PD-1 expressors lysed less efficiently. Six reactive clones, selected for their distinct functional attributes, shared the same TCR sequences, confirming a common T cell precursor origin. In a second patient with metastatic biliary tract adenocacarcinoma, 2 out of 604 screened CD8+ TIL clones were reactive to autologous cancer cell lines with specificity by lysis, upregulation of 4-1BB, and IFN-g secretion. The two clones did not share the same TCR and had distinct level of reactivity.

Conclusions: CD8+ effector memory TIL found in fresh GI cancer metastases expressed higher levels of 4-1BB and PD-1 when compared to autologous PBL. In two cases for which we could generate new cancer cell lines, we isolated CD8+ TIL clones with specific autologous tumor recognition. IFN-g secretion alone appears insufficient for selecting tumor-specific TIL in GI cancer metastases.

Key Words: Cellular immunity, Colorectal cancer, Tumor infiltration lymphocytes.

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Gc PROTEIN-DERIVED MACROPHAGE ACTIVATING FACTOR (GcMAF) CAN ERADICATE A VARIETY OF CANCERS INDISCRIMINATELY

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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 lysoalkylphospholipids 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 the tri-saccharide of serum Gc protein (known as vitamin D-binding protein) with the inducible β-galactosidase (Bgl) of inflammation-primed B cells and the Neu-1 sialidase of T cells to yield the macrophage activating factor (MAF), the protein with N-acetylgalactoamine as the remaining sugar. Thus, Gc protein is the precursor for the principal 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. Thus, serum Nagalase activity is proportional to tumor burden and serves as excellent diagnostic and prognostic indices. Stepwise treatment of purified serum Gc protein with immobilized β-galactosidase and sialidase generates probably the most potent MAF (termed GcMAF) that produces no side effect in humans. Macrophages activated by GcMAF develop an enormous variation of receptors that recognize the abnormality (TAA: tumor associated antigen) in malignant cell surface and are highly tumoricidal to a variety of cancers indiscriminately. Types of cancer so far tested are breast, prostate, colorectal, stomach, liver, lung, esophagus, kidney, bladder, cervix, ovary, head/neck cancers, melanoma, fibrosarcoma, leukemia, glioblastoma and mesothelioma. When human macrophages/monocyes were treated in vitro with GcMAF (100 picogram/ml) for 3 hr and a breast cancer cell line MCF-7 was added with effector/target ratio of 3, 58% and 85% of MCF-7 cells were killed in 4 hr and 18 hr incubation, respectively. When non-anemic adenocarcinoma (such as metastatic breast, prostate and colorectal cancer) patients were intramuscularly administered with 100 ng GcMAF/week, their tumors were eradicated in 16-25 weeks. When MCF-7 cells were preincubated with the above Gc-MAF-treated breast patient serum for 2 hrs and added to the Gc-MAF-activated macrophages which have developed a large amount of Fc-receptors, approximately 86% of MCF-7 cells were killed in 4 hr incubation, indicating development of target specific immunity.

Key Words: Breast cancer, Macrophages, Tumor associated antigen.

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FULLY AUTOMATED GENERATION OF MULTI-VIRUS-SPECIFIC T-CELLS FOR ADOPTIVE IMMUNOTHERAPY

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Adoptive transfer of T-lymphocytes with antigen-specificities for one or several viruses is a promising strategy to treat or prevent opportunistic infections in immunocompromised patients. Human adenovirus, Epstein-Barr-virus, and cytomegalovirus infections are frequently observed and often cause life-threatening complications following allogeneic stem cell transplantation. Multi-virus-specific CD4+ and CD8+ T-cells can rapidly be generated by a short-term antigen-specific restimulation of peripheral blood cells with a combination of peptide pools covering the complete viral antigens and a subsequent isolation using the Cytokine Capture System IFN-gamma (CCS).

A novel cell processing device was developed, which performs all steps of the CCS procedure, i.e. restimulation, magnetic enrichment, and potentially in vitro expansion fully automated under sterile conditions. All components for the generation of the cellular product including the cellular starting material (e.g. leukapheresis or PBMC), antigen(s), reagents, buffer, and media are connected to a sterile single-use functionally closed tubing set via sterile filters or docking technique. Cell processing can run overnight and the isolated cells might be used directly after magnetic enrichment or after an additional phase of in vitro expansion. Using this cell processing device, IFN-gamma secreting multi-virus-specific T-cells can be enriched to the same purity as with the semi-automated procedure. Cell loss is markedly reduced, leading to an increased yield of IFN-gamma positive cells. An improved viability was observed resulting in better expansion rates. To confirm functionality of the enriched multi-virus-specific T-cells and their composition in respect to single antigen specificities, cells were analyzed for the IFN-gamma response after expansion and restimulation with single peptide pools or with a mixture of the antigenic peptide pools. Restimulation with an antigen mixture resulted in a high proportion of T-cells re-expressing IFN-gamma. Interestingly, restimulation with single peptide pools revealed the relative frequencies of T-cells specific for each single antigen were comparable before and after the co-enrichment and co-expansion process, showing the protocol works for T-cells independent of their pathogen specificity. In conclusion, the automation enables an easy, safe, fast and robust generation of antigen-specific T-cells for adoptive immunotherapy with minimal manual intervention, reduced workload and also simplified clean-room requirements.

Key Words: CD4+ T cells, CD8+ T cells, Cytokine.

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COPPER-64 BASED PET FOR WHOLE-BODY BIODISTRIBUTION OF ADOPTIVELY TRANSFERRED T CELLS

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Adoptive T Cell Therapy (ACT) shown to be effective against tumors depend on the ability of genetically modified T cells to traffick to the disease sites and hence require a means to assess their biodistribution. Current methods include serial sampling and conducting quantitative PCR or flow cytometry; both of which are invasive and do not provide whole-body distribution. Genetic variants of T cells may include transgenes for ^{18}F-based PET imaging but its use is limited to pre-clinical trials only.

Standard operating procedures currently in clinical trials for ACT of B-cell lymphoma were used to genetically modify donor derived primary T cells by electroporation of the ***Sleeping Beauty*** (SB) transposon/transposase system. T cells were genetically modified to express chimeric antigen receptors (CAR) to impart specificity for CD19-antigen expressed on B-cell lymphomas and expanded ***ex vivo*** on CD19$^+$ clinical-grade master cell bank of artificial antigen presenting cells (aAPC). To non-invasively determine the whole-body biodistribiution of infused T cells, we labeled expanded CAR$^+$ T cells with positron emitter, copper-64 ($^{64}Cu^{+2}$), before infusion in immunocompromised (nu/nu) mice. This was done by electrotransfer of gold nanoparticles (GNP) functionalized with $^{64}Cu^{+2}$ and polyethyleneglycol (GNP-$^{64}Cu^{+2}$/PEG2000). Firefly Luciferase (ffLuc) transgene was expressed for validation studies.

Whole-body biodistribution of infused CAR$^+$ ffLuc$^+$ GNP-$^{64}Cu^{+2}$/PEG2000$^+$ was observed using μPET/CT (micro positron emission tomography/computed tomography) scan over the period of 12 hrs. Correlation of PET data with bioluminescent imaging (BLI) provides 'proof of principle' for the use of $^{64}Cu^{+2}$ labeled genetically modified T cells for their ***in vivo*** tracking and to determine their therapeutic index. ***Post-mortem*** assessment of lung-to-liver biodistribution ratio of radioactivity also revealed that packaging GNPs into T cells helped their delivery to the lungs as compared to being trapped in the liver when GNPs were infused directly. This approach also has clinical appeal as it builds upon currently ongoing clinical trials of adoptive T cell therapy for CD19$^+$ B-cell lymphoma (IND 14193 & 14577). Currently, work is in progress to investigate alternate mechanisms for delivery of nanoparticles and PET imaging agents into ***ex vivo*** expanded T cells that may improve their ***in vivo*** persistence.

Key Words: Adoptive therapy.

Presenting author underlined. *Primary author in italics.*

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EGFR-MEDIATED LYSIS OF GLIOMA CELL LINES BY GENETICALLY MODIFIED T CELLS

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Diffuse Intrinsic Pontine Glioma (DIPG) is an inoperable and nearly uniformly fatal tumor that overexpresses wild type epidermal growth factor receptor (EGFR). We seek to extend antibody therapy for EGFR to T-cell therapy by introducing a chimeric antigen receptor (CAR) to redirect the specificity of autologous T cells to EGFR. An EGFR-specific CAR was generated by fusing the specific region (scFv) of an EGFR-specific antibody to T-cell activation domains CD28 and CD3ζ, expressed as ***Sleeping Beauty*** (SB) transposon, and inserted by transposition into primary peripheral blood mononuclear cells (PBMC). T cells expressing CAR were propagated on irradiated artificial antigen presenting cells (aAPC) modified to express EGFR and added recursively to the culture every seven days in the presence of IL-2 and IL-21. EGFR-specific T cells numerically expanded 1000-fold over 28 days to yield a population of primarily CAR^+CD8^+ T cells with effector-memory phenotype ($CCR7^{neg}$ $CD45RO^+$) expressing perforin and granzyme B. CAR^+ T cells were specifically activated in the presence of targets expressing EGFR, but not targets expressing irrelevant antigen, which resulted in the production of IFN-γ and TNF-α, but not IL-4, IL-10 or TGF-β. CAR^+ T cells were able to lyse ~95% of $EGFR^+$ glioma cell lines U87MG, T98G, and LN18 in a 4 hour ^{51}Cr release assay, but not tumor cells expressing irrelevant antigen. Lysis of $EGFR^+$ targets was blocked by an EGFR antibody in a dose-dependent manner, indicating that the CAR is mediating lysis through recognition of EGFR on tumor targets. In aggregate, these studies demonstrate that CAR^+ T cells are capable of EGFR-dependent lysis of tumor cells. Given our ability to use the SB system and aAPC to generate CAR^+ T cells to target CD19 on B cell malignancies in the clinic, we will translate these data to clinical trials.

Key Words: Chimeric receptors.

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A HIGH-THROUPUT ENGINEERING AND IN VIVO SELECTION SYSTEM FOR ESTABLISHING OPTIMAL AFFINITIES OF T CELL RECEPTORS FOR ANTI-CANCER RESPONSES

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Adoptive T cell therapies have shown significant promise in the treatment of cancer. Further advantages have been realized by the introduction of tumor antigen-specific T cell receptor (TCR) genes into T cells activated ex vivo. This approach could, in principle, overcome central tolerance mechanisms that prevent efficient responses by the endogenous T cell repertoire. To date, however, the rules for binding affinity that govern a TCR's ability to optimally recruit CD8+ and CD4+ T cells to a cancer are unknown. Investigation of these issues is complicated by the need to test each TCR for efficacy on a one-by-one basis. To more rapidly query the impact of diverse TCR binding properties, we developed a high-throughput platform for examining T cell targeting of cancer by constructing libraries of structurally related TCRs with a wide range of affinities for a model tumor epitope. The library was characterized via T cell display in vitro, and the variants with the best binding to the soluble target epitope were identified. Administration of CD8+ or CD4+ T cells transduced with this library mixture of TCRs provided effective T cell therapy of cancer in a mouse model. High-throughput sequencing of individual TCR transcripts present in mouse tumors and tissues allowed rapid determination of those TCR sequences which were selected in vivo for optimal T cell infiltration into tumors and peripheral T cell persistence. Our results provide insight into the relationship between TCR binding and adoptive T cell effectiveness against tumors, and they can guide improvements to adoptive T cell strategies.

Key Words: TCR, Affinity, Engineering.

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CD19-TARGETED T CELLS EXPRESSING A SECOND-GENERATION CD28-BASED CHIMERIC ANTIGEN RECEPTOR EVADE CTLA-4 INHIBITION

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Human T cells that express a chimeric antigen receptor (CAR) composed of an antibody-derived scFv fragment linked to the CD3 zeta chain, can recognize and kill tumor cells expressing the targeted membrane antigen. However, costimulatory signals are needed for CAR-targeted T cells to avoid anergy, to be more fully activated and sustain proliferation. CD80 and CD86 are the prototypical costimulatory molecules, which bind the CD28 receptor constitutively expressed at the T cell surface. We previously reported that T cells expressing a CAR along with CD80 undergo auto-costimulation. We also showed that CD19-targeted T cells harboring a second generation CAR (1928z), which additionally comprises the CD28 cytoplasmic domain, promote higher tumor rejection rate than T cells expressing a first generation CAR (19z1) containing only the zeta chain-signaling domain.

In this study, we asked if 1928z expressing T cells displayed the same anti-tumor capacity as T cells expressing 19z1 and CD80. CD80 and CD86 indeed activate CTLA-4, a strong inhibitory molecule, which is recruited at the T cell surface shortly after activation. We compared the proliferation and anti-tumor abilities of 19z1+, 19z1-CD80+ and 1928z+ T cells in an aggressive pro-B cell leukemia model (NALM-6). Upon binding to CD19, 1928z+ T cells ini-

tially secreted IL-2 and showed enhanced proliferation and survival. Using in vivo bioluminescence imaging to monitor tumor burden over time, we observed a 10-fold reduced tumor progression rate in NALM-6 bearing NOD/SCID/IL2Rγnull mice receiving 1928z+ T cells over those treated with 19z1+ or 19z1-CD80+. The median survival was 3-fold increased. We then investigated if CTLA-4 -mediated inhibition signal triggered by CD80 may account for this difference. We observed that CTLA-4 was expressed at higher level at the cell surface by 19z1-CD80+ T cells than 1928z+ T cells after antigen activation. Furthermore, 19z1-CD80+ T cells expressing an anti-CTLA-4 shRNA showed significantly increased in vivo anti-tumor abilities, reaching the survival rate obtained with 1928z+ T cell treatment. These results strongly suggest that 1928z+ T cells, which prolong survival of animals bearing aggressive leukemia, are less sensitive to CTLA-4 inhibition that CD80-costimulated T cells.

Key Words: Adoptive therapy, Chimeric receptors, CTLA-4.

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SPECIFICITY OF HUMAN GAMMA DELTA T CELLS CAN BE RE-DIRECTED TO CD19 WHILE AVOIDING UNWANTED ALLOGENEIC RESPONSES

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T cells can be separated into two subsets based on their T-cell receptor (TCR) isotype, of which the αβ T cells (TCRαβ) and γδ T cells (TCRγδ) represent ~95% and ~5% of the peripheral T-cell pool, respectively. Chimeric antigen receptors (CAR) are used to re-direct the specificity of T cells to tumor associated antigens, e.g. the B-cell surface antigen CD19, independent of the interaction between TCR and major histocompatibility complex (MHC). To transition from infusing donor-derived T cells, we are seeking an approach to limit deleterious allogeneic immune responses. TCRγδ does not recognize peptides within the context of MHC making γδ T cells less apt to initiate graft-versus-host-disease (GvHD) compared with αβ T cells. Thus, we hypothesized that the cytolytic capacity of γδ T cells can be combined with the targeting by CD19-specific CAR to kill B-cell malignancies without allogeneic responsiveness. ***Sleeping Beauty*** transposition was used for non-viral gene transfer of a CD19-specific CAR into PBMC, and CAR^+ αβ and γδ T cells were expanded on K562-derived artificial antigen presenting cells (aAPC) modified to express CD19 and T-cell co-stimulatory molecules. As controls, bisphosphonates and anti-CD3 (OKT3) antibody were used for the numeric expansion of $CAR^{neg}TCR\gamma\delta^+$ and $CAR^{neg}TCR\alpha\beta^+$ T cells, respectively. Expression of the CD19-specifc CAR resulted in superior expansion of γδ T cells on $CD19^+$ aAPC when compared to bisphosphonates, and $CAR^+TCR\gamma\delta^+$ T cells were readily produced in kinetics similar to $CAR^+TCR\alpha\beta^+$ T cells. Both $CAR^+TCR\alpha\beta^+$ and $CAR^+TCR\gamma\delta^+$ T cells exhibited re-directed specificity for CD19 with similar CAR-dependent cytolytic capability and production of pro-inflammatory cytokines (IFNγ and TNFα). To address the potential for alloreactivity, we note that $CAR^+TCR\gamma\delta^+$ T cells exhibited inferior allogeneic responses compared to $CAR^+TCR\alpha\beta^+$ T cells when challenged with allogeneic monocyte-derived dendritic cells. There was a difference in homing molecule expression between $CAR^+TCR\alpha\beta^+$ and $CAR^+TCR\gamma\delta^+$ T cells, which suggests that the latter may have access to desired tissue locations. $CAR^+TCR\gamma\delta^+$ T cells propagated on $CD19^+$ aAPC were not restricted to Vδ2 lineage seen with bisphosphonates and include γδ T cell subsets appealing for cancer immunotherapy. To test the ***in vivo*** anti-tumor effect of CAR^+ T cells, immunocompromised mice were engrafted with $CD19^+$ NALM6 cells and challenged with CAR^+ T cells. $CAR^+TCR\gamma\delta^+$ T cells achieved a more potent anti-tumor effect than $CAR^+TCR\alpha\beta^+$ T cells in acute therapy phases. In conclusion, this study reports a clinically-appealing approach to generate large numbers of allogeneic $CAR^+TCR\gamma\delta^+$ T cells specific for B-cell malignancies with minimal risk of GvHD.

Key Words: Adoptive therapy, GVHD, Leukemia.

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ADOPTIVE T CELL THERAPY FOR PATIENTS WITH ADVANCED MALIGNANT MELANOMA - EXPERIENCE FROM A DANISH TRANSLATIONAL RESEARCH INSTITUTION

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Background: T cell therapy is based on isolation of tumour infiltrating lymphocytes (TIL) from autologue tumour, their in vitro activation and expansion and the reinfusion of these cells into a lymphodepleted patient. This treatment has in a few other countries successively been given to patients with advanced malignant melanoma (MM) and an impressive 50% response rate has been observed. Here we report the experience from a Danish Translational Research Center.

Methods: A pilot trial including patients with metastatic MM, PS ≤1, age <70, measurable and progressive disease and no brain metastases. Inclusion is ongoing.

The treatment consists of high-dose chemotherapy day 1-7, TIL infusion day 8 and 14 days of low-dose interleukin-2. Primary objectives are safety and toxicity, secondarily immunological and clinical responses, time to progression and overall survival will be assessed.

Results: Six of planned 14 patients with advanced MM have been enrolled and treated according to the protocol. Adverse events include expected myelosuppression, gastrointestinale symptoms and general fatigue, but also hypertension, tachycardia, fever, chills and low sodium levels were observed.

One patient obtained a partial response shortly after treatment and is now in complete remission 20 months after therapy. Two patients had stable disease at 1st evaluation but progressed after

4.5 and 5 months respectively. Two patients progressed shortly after treatment and one patient just finished treatment and awaits evaluation.

Five patients have gone through surgery but have not been treated because of rapid disease progression, development of brain metastases and/or death. Another two patients have been included in the trial and awaits treatment/surgery.

Analyses of immune responses are ongoing; results will be available for presentation at the meeting.

Conclusion: The observed adverse events have been manageable and acceptable. Five patients have been clinically evaluated so far - one with a complete response.

Key Words: Adoptive therapy.

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DESIGN AND PRELIMINARY RESULTS OF A CLINICAL TRIAL WITH 4-1BB-STIMULATED "ECCE" TIL FOR PATIENTS WITH ADVANCED MELANOMA

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Adoptive cell therapy with autologous tumor-infiltrating lymphocytes (TIL) can achieve up to 72 percent objective responses including durable complete regressions in patients with refractory metastatic melanoma. However, the complexity and unpredictability of clinical-scale TIL growth limit the widespread use of this approach. TIL are initiated from melanoma deposits and expanded by in vitro culture to greater than 1010 T cells for therapy. We determined that CD137 (4-1BB) co-stimulation delivers proliferative and survival signals that result in a simplified and more reliable TIL production process. TIL were stimulated by "engineered cells for costimulatory enhancement" (ECCE) comprised of K562 cells transduced to express CD64 (high affinity Fc receptor) and CD137L (4-1BBL). Preclinical experiments demonstrated that ECCE provision significantly improved TIL growth from tumors, including tumors for which TIL expansion was challenging (30/37 vs 13/37, p=0.0001). Also the addition of ECCE to the rapid expansion protocol (REP) reduced the peripheral blood mononuclear "feeders" required by ≥80 percent yet equivalently expanded TIL compared to current methods (1038±89 vs 964±117, n=13, p=0.3). Based on these results, a clinical trial was developed using ECCE-grown TIL to treat metastatic melanoma patients. Cells were expanded from an ECCE master cell bank, γ-irradiated, vialed, and cryopreserved according to current good manufacturing practices (cGMP). New qualification assays required for use as an ancillary cell product in clinical TIL production were developed. These assays included demonstrating lack of proliferation of the ECCE ancillary cell bank at a sensitivity of less than 0.001% and quantifying ECCE elimination from the TIL product at a sensitivity below 0.05%. ECCE TIL are being evaluated in a two arm study for safety and efficacy: The first arm targets patients for whom TIL cannot be grown by conventional methods. Initially 9 patients are enrolled on this arm and if one or more has a clinical response, accrual will continue to 24 patients, targeting a 25% goal for objective response. Included in cohort one was a patient who received TIL derived exclusively from core needle biopsies because no resectable tumor was available. The second arm targets patients with a conventional TIL culture, but uses an optimized ECCE REP to expand the cells to large numbers for treatment. Initially 18 patients are enrolled and if three or more have a clinical response, accrual will continue to 35 patients, targeting a 30% goal for objective response. The preliminary clinical and immunological results of this protocol will be discussed.

Key Words: Adoptive therapy, Melanoma, Phase II.

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DISSECTING THE EFFECTS OF TIL THERAPY IN MELANOMA PATIENTS

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Adoptive cell therapy using autologous tumor infiltrating lymphocytes (TIL) has emerged as a highly promising treatment for patients with metastatic melanoma. However, the clinical effects of TIL therapy have not been evaluated in a randomized trial and our understanding of the effects of TIL therapy on the melanoma-specific T cell repertoire is still limited. To address the first issue we will carry out a first randomized phase II clinical trial at the NKI-AVL comparing TIL therapy with standard therapy. Data on the first patients treated in the phase I part of this trial will be presented. To address the second issue, we have utilized a combination of two novel technologies - MHC multimer generation by ligand exchange and multiplexed flow cytometry by combinatorial coding - to measure T cell reactivity against a panel of over 140 shared melanoma-associated HLA-A2 epitopes. Using this approach, we assessed the composition of pre-infusion TIL from 34 melanoma patients treated in the US and Israel, but also the effect of TIL therapy on the melanoma-specific T cell repertoire in peripheral blood. The data obtained demonstrate that individual tumor-infiltrating lymphocyte cell products from melanoma patients contain unique patterns of reactivity against shared melanoma-associated antigens, but that the combined magnitude of these responses is surprisingly low. Importantly, TIL therapy increases the breadth of the tumor-reactive T cell compartment in vivo, and T cell reactivity observed post-therapy can almost in full be explained by the reactivity observed within the matched cell product. These results establish the value of high-throughput monitoring for the analysis of immuno-active

therapeutics such as TIL, and suggest that the clinical efficacy of TIL therapy may be enhanced by the preparation of more defined tumor-reactive cell products.

Key Words: Adoptive therapy, Tumor infiltration lymphocytes.

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19F MRI CELLULAR TRACER PRESERVES THE DIFFERENTIATION POTENTIAL OF HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSC) have numerous applications including immune reconstitution, enzyme replacement, regenerative medicine and immunomodulation. The trafficking and persistence of these cells after administration is a question fundamental to the future of alternative therapeutic applications of HSC. While various contrast agents have been used to track cellular therapeutics, the impact of cellular labels on HSC function has not previously been studied, independently of therapeutic outcome, in vivo. Here we describe the labeling of human CD34+ HSC with a novel self-delivering perfluorocarbon (PFC) emulsion. This magnetic resonance imaging (MRI) tracer agent has been found to lack mutagenicity in multiple in vitro studies. A comparison of unlabeled and PFC-labeled human HSC in in vitro colony forming assays resulted in equal numbers of total colony forming units (CFU), as well as individual CFU types, indicating that labeling did not alter multipotency. In parallel, in vivo tests of pluripotency and reconstitution studies in mice with labeled murine BM HSC resulted in equivalent development of CFU-spleen (a measure of HSC progenitor activity) and the reconstitution of both lymphoid and myeloid compartments. The lack of interference in these highly complex biological processes both in vitro and in vivo following PFC labeling, provides strong evidence that the therapeutic potential of the HSC is likely to be maintained. These data support the safety and utility of using PFC tracers for clinical in vivo trafficking of human stem cells.

Key Words: Cell trafficking.

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IMPROVED IN VIVO PERSISTENCE OF CD19-SPECIFIC T CELLS EXPRESSING A MEMBRANE-BOUND FORM OF IL-15

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Clinical responses in adoptive immunotherapy are associated with in vivo expansion and persistence of the transferred antigen-specific T cells. Therapeutic efficacy is hampered by a lack of persistence of infused T cells. Interleukin (IL)-2 is used in adoptive therapies to stimulate T cell expansion and promote their survival. Systemic administration of IL-2 is limited as its use is associated with toxicity and expansion of regulatory T cells. IL-15 may be a more suitable cytokine as it promotes memory CD8+ T cell survival and can re-establish the functionality of T cells. To deliver localized IL-15 signaling to T cells, we generated a version of IL-15 as a membrane-bound molecule (mbIL15). The mbIL15 construct was electro-transferred with a CD19-specific CAR (on day 0) into primary human T cells as Sleeping Beauty DNA transposon plasmids. Numeric ex vivo expansion of these genetically modified T cells was achieved by co-culture on CD19+ artificial antigen presenting cells derived from K562, but without additional soluble cytokine supplementation. Preferential outgrowth of T cells expressing both mbIL15 and CAR was attained, while CAR+ T cells receiving no soluble cytokine supplementation did not undergo expansion. In our culture system, mbIL15-modified T cells acted in synergy with exogenous IL-21 to achieve superior expansion and maintained a mixture of effector and central memory phenotypes. Signaling through the IL-15 receptor complex in mbIL15+CAR+ T cells was validated by phosphorylation of STAT5. The mbIL15+CAR+ T cells demonstrated redirected specific lysis of CD19+ tumor targets equivalent to the standardly cultured CAR+ T cells. Adoptive transfer of modified T cells into immunodeficient mice demonstrated stable persistence of mbIL15+CAR+ T cells greater than 35 days, whereas CAR+ T cells were not detected past 4 days. In mice bearing CD19+ malignancy, mbIL15+CAR+ T cells demonstrated both homing and anti-tumor effects. These data demonstrate that mbIL15 can be expressed by CAR+ T cells to enhance their proliferation and in vivo persistence without the need for exogenous cytokine support. The use of this fusion molecule: (i) provides stimulatory signals via STAT5 leading to augmented in vivo T cell persistence while maintaining tumor-specific functionality, (ii) eliminates the need for IL-2 for T-cell expansion and persistence, and (iii) mitigates the need for clinical-grade IL-15. These results have implications for the design of adoptive immunotherapy clinical trials to evaluate whether mbIL15+CAR+ T cells can improve therapeutic potential.

Key Words: Adoptive therapy, Chimeric receptors, Cytokine.

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SIMPLIFIED METHOD OF THE GROWTH OF TUMOR INFILTRATING LYMPHOCYTES (TIL) IN GAS-PERMEABLE FLASKS TO NUMBERS NEEDED FOR PATIENT TREATMENT

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Adoptive cell therapy (ACT) of metastatic melanoma with autologous tumor infiltrating lymphocytes (TIL) is clinically effective, but TIL production can be challenging. Initial TIL culture and rapid expansion involves 4 to 6 weeks of culture in 24-well plates, tissue culture flasks and bags. Here we describe a simplified method for initial TIL culture and rapid expansion in gas-permeable flasks. TIL

were initially cultured from tumor digests and fragments in 40 mL capacity flasks with a 10 cm2 gas-permeable silicone bottom, G-Rex10. A TIL rapid expansion protocol (REP) was developed using 500 mL capacity flasks with a 100 cm2 gas-permeable silicone bottom, G-Rex100. TIL growth was successfully initiated in G-Rex10 flasks from tumor digests from 13 of 14 patients and from tumor fragments in all 11 tumor samples tested. Enough TIL for REP could be obtained from one G-Rex10 flask (tumor digest) or 3 G-Rex10 flasks (tumor fragments) rather than two 24-well plates. TIL could then be expanded to 8-10 billion cells in a two-step REP which began by seeding 5 million TIL and 500 million irradiated peripheral blood mononuclear cells as feeder cells into a G-Rex100 flask. After 7 days of culture the cells were split and divided equally into 3 G-Rex100 flasks for the second step of TIL REP. This two-step G-Rex100 flask TIL REP was successful in 12 of 14 patients studied. To obtain the 30 to 60 billion cells used for patient treatment we seeded 6 G-Rex100 flasks with 5 million cells and later transferred the TIL into 18 G-Rex100 flasks. Large scale TIL REP in gas-permeable flasks requires approximately 9 to 10 liters of media, about 3 to 4 times less than other methods. TIL expanded using the optimized protocols described here have been safely administered to patients in ongoing clinical trials, and can mediate tumor regressions and objective clinical responses. In conclusion, TIL initiation and REP in gas-permeable G-Rex flasks require fewer total vessels, less media, less incubator space and less labor than initiation and REP in 24-well plates, tissue culture flasks and bags. TIL culture in G-Rex flasks will facilitate the production of TIL at the numbers required for patient treatment at most cell processing laboratories.

Key Words: Adoptive therapy, Tumor infiltration lymphocytes.

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CD56 EPITOPE ESCAPE ON T CELLS ALLOWS FOR NUMERIC EXPANSION OF CD56-SPECIFIC T CELLS WITHOUT AUTOLYSIS

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The expression of some candidate tumor-associated antigens (TAAs) on T cells precludes the development of TAA-specific T-cell based therapies. One such antigen, CD56, is expressed on many malignancies, yet CD56 upregulation by activated T cells could lead to self targeting, i.e. autolysis. We developed a second generation CD56-specific chimeric antigen receptor (CAR, designated CD56RCD28) and show that these CAR^+ T cells can be propagated in a CAR-dependent fashion and have CD56-specific cytotoxicity against a panel of $CD56^+$ tumor cell lines including neuroblastoma, small cell lung carcinoma, glioma, and NK cell leukemia. Surprisingly, $CD56RCD28^+$ T cells can co-express the CD56 antigen. We therefore investigated if these $CD56RCD28^+$ T cells co-expressing the CD56 TAA were undergoing autolysis and if not, why not? Primary human T cells were selectively propagated on -irradiated $CD56^+$ artificial antigen presenting cells (aAPCs, added every 7 days) in the presence of exogenous recombinant human IL-2 and IL-21. We first evaluated the ability of $CD56RCD28^+$ T cells to lyse autologous $CD56^+$ lymphocytes with and without the CAR in a chromium release assay (CRA). $CD56^+CD56RCD28^+$ T cells were not significantly lysed consistent with resistance to autolysis, while autologous $CD56^+CAR^{neg}$ T cells, $CD56^+CD19RCD28^+$, and $CD56^+$NK cells were significantly lysed. $CD56RCD28^+$ T cells were also not significantly activated in response to $CD56^+CD56RCD28^+$ T cells as assessed by CD107a expression, CD69 upregulation, and IFN-γ production. In sum, these data infer that $CD56^+CD56RCD28^+$ T cells evade autolysis and avoid recognition by CD56RCD28. Our CD56-specific CAR derives its specificity from the monoclonal antibody (mAb) clone N901, used in clinical trials. Therefore we investigated CD56 expression on CAR^+ T cells using N901 and the CD56-specific mAb clone B159. We observed that N901 expression, but not B159 expression was reduced on $CD56RCD28^+$ T cells. Control CD19-specific CAR^+ T cells had no reduction in either N901 or B159 expression. To conclude, our data demonstrate that we can expand out functional CD56-specific CAR^+ T cells with the ability to co-express the CD56 antigen and the capacity to evade autolysis. These data shed light on escape from CAR recognition and highlight that rationale selection of mAbs for the construction of CARs can be used to target tumor and not self.

Key Words: Adoptive therapy, Chimeric receptors, Neuroblastoma.

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REDIRECTED ANTI-TUMOR ACTIVITY OF PRIMARY HUMAN LYMPHOCYTES TRANSDUCED WITH A FULLY-HUMAN ANTI-MESOTHELIN CHIMERIC RECEPTOR

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Cancer regression by gene-modified T cells bearing chimeric antigen receptors (CAR) of mouse origin has been limited by the induction of transgene immunogenicity resulting in poor persistence and function in vivo. The development of functionally-active CAR of human origin can address this issue. Here, we constructed and evaluated a fully-human anti-mesothelin CAR comprised of a human mesothelin-specific single-chain antibody fragment (P4 scFv) coupled to an intracellular TCR CD3ζ chain with or without a CD28 costimulatory domain. Primary human T cells expressing P4 CAR specifically produced proinflammatory cytokines, degranulated and exerted potent cytolytic functions when co-cultured with mesothelin-expressing tumors in vitro. P4 CAR T cells also mediated bystander killing of mesothelin-negative cancer cells during co-culture. Furthermore, soluble tumor-secreted or recombinant mesothelin protein did not abrogate mesothelin-specific CAR reactivity even at supraphysiological levels. Importantly, adoptive transfer of P4 CAR-expressing T cells mediated the regression of large, established tumors in the presence of soluble mesothelin in a xenogenic model of human ovarian cancer when administered either intratumorally or intravenously. Thus, primary human T cells expressing fully-human anti-mesothelin CAR efficiently kill mesothelin-expressing tumors in vitro and in vivo and have the potential to overcome the issue of transgene immunogenicity that has limited CAR T cell trials that utilize scFvs of mouse origin.

Key Words: Adoptive therapy, Chimeric receptors, Tumor associated antigen.

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TUMOR LACTIC ACIDOSIS SUPPRESSES CTL FUNCTION BY INHIBITION OF P38 AND JNK/C-JUN ACTIVATION

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We and others were able to show functional deficits as well as phenotypic aberrations in tumor infiltrating lymphocytes isolated from tumor tissues. Moreover, published data indicate that not only the natural antitumor immune reponse is compromised by the tumor milieu, but adoptively transferred T cells also lose their function after infiltration into the tumor. A detailed understanding of the tumor milieu and the factors which cause effector T cell (TEff cell) impairments is required to allow the development of counter measures to improve the functional capacity of TEff cells used for adoptive T cell therapy. To elucidate suppressive factors we mimicked the tumor milieu in vitro and challenged the functional capacity of TEFF cells within this milieu. It was observed that soluble factors produced by high density tumor cell culture were sufficient to completely suppress pMHC stimulated IFNγ production of TEFF and diminish the capacity for lytic granule exocytosis. High levels of extracellular lactate with concentrations reaching up to 40 mM and concomitant extracellular acidification (pH 6.4 to 6.8) are found in the milieu of solid tumors. Supernatants of tumor cells, which induced functional CTL inhibition, contained lactate in concentrations around 20 mM and had pH values between 6.4 and 6.6, thus reflecting in situ tumor conditions. Supplementing whole medium with 20 mM lactic acid mimicked the effect of high density soluble tumor cell supernatant in that it completely suppressed IFNγ production and reduced degranulation capacity. No inhibitory effects were seen when medium was supplemented with sodium lactate that had neutral pH. TCR signaling is a prerequisite for T cell function. CD3 stimulated phosphorylation of MAP kinases JNK and donwnstream c-Jun, and p38 was diminished in lactic acid containing medium. Activation of proximal signaling molecules (LCK, LAT, ZAP70) was not affected by the presence of lactic acid, nor was the phosphorylation of ERK or AKT.

Removing CTLs from the lactic acid milieu allowed rapid recovery of their functional ability. Concomitant with functional recovery was an increased phosphorylation of p38, JNK and c-Jun. Moreover, neutralization of the acidic milieu restored IFNγ production and degranulation, despite the continuous presence of lactate. This is an important observation as it suggests that therapeutic interventions neutralizing acidosis could help prevent effector phase inhibition and improve antitumor activity of adoptively transferred CTL.

Key Words: Adoptive therapy, CD8+ T cells, Tumor microenvironment.

Presenting author underlined. *Priminary author in italics.*

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HUMAN ANTI CANCER CYTOKINE INDUCED KILLER CELLS AND THEIR INTERACTION WITH THE HOST IMMUNE SYSTEM

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Cytokine-induced killer cells are in vitro expanded cells used in adoptive therapy for the treatment of solid and hematopoietic tumors. They express the T cell markers CD3 and CD56 and lack the cytotoxicity machinery of natural killer (NK) cells. Several papers have described the interactions between cytokine-induced killer cells and tumor targets. We focused our attention on the specific interplay between cytokine-induced killer cells and peripheral blood mononuclear cells in order to elucidate the possible consequences of this interaction.

Cytokine induced killer cells were generated from peripheral blood mononuclear cells of 5 healthy donors by 21 days ex vivo expansion after priming with interferon gamma and monoclonal anti-CD3 antibody in medium supplemented with interleukin-2. After the expansion, cells were stained with CFSE and co-cultured with autologous peripheral blood mononuclear cells for 5 h. The co-cultures were then stained with anti-CD14 antibody for monocytes, anti-CD19 antibody for B lymphocytes, anti-CD56 antibody as activation marker, anti-CD3, anti-CD4 and anti-CD8 for lymphocytes subpopulations, and analyzed by means of Imagestream and cytofluorimetric techniques.

We observed that after the 5 h incubation, the percentage of CFSE-CD14 double positive cells increased in a ratio dependent manner. The physical interaction was confirmed by Imagestream technique. We detected a small number of double positive CFSE-CD19 cells and we noticed a decrease in the total number of CD19 cells suggesting a possible specific cytotoxic activity against B cells.

We confirmed this in vivo noticing that two patients undergoing CIK therapy showed a transient reduction in number of circulating CD19.

The direct toxicity versus healthy circulating B cells could explain why this therapy leads to the maximum therapeutic benefit in the treatment of hematological malignancies. We noticed as well in vitro a CD56 increased expression on CIK cells that could be explained by the increased activation after monocyte contact. Further investigation is required to elucidate whether this activation could be different in the presence of suppressive monocytes. Our results could be of crucial relevance especially in the setting of solid tumor where the strong immunosuppressive context could negatively influence the activation status of the adoptively infused cells.

Key Words: Adoptive therapy.

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GENERATION AND OPTIMIZATION OF A CHIMERIC ANTIGEN RECEPTOR AGAINST CD22: A NEW IMMUNOTHERAPEUTIC AGENT FOR TREATING B LINEAGE LEUKEMIA AND LYMPHOMA

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CD22 is expressed on a number of hematologic malignancies. A recombinant immunotoxin composed of an anti-CD22 Fv fused to a 38 kDa fragment of Pseudomonas exotoxin A (Moxetumomab pasudodtox, HA22) is currently being evaluated in the treatment of B cell malignancies. We used the Fv sequence to construct a series of chimeric antigen receptors (CARs) to determine the optimal affinity, domain structure, and signaling required for optimal anti-leukemic activity. The original anti-CD22 binding domain, BL22, or a high-affinity domain, HA22, were fused to transmembrane and signaling sequences derived from the TCR zeta-chain, CD28, and CD137. In some constructs we extended the binding domain away from the membrane using constant domains from IgG (CH2CH3). Retroviral gene vectors were used to transduce activated primary T cells with CAR constructs. We found: HA22 Fv, 2 as opposed to 3 signaling motifs, and non-CH2CH3 containing CARs were superior in short-term in vitro tumor cell cytolysis assays, indicating that Fv affinity, signaling, and 3-D structure all impact the anti-leukemic effectiveness of CARs. Finally, direct comparison to CD19-specific CAR showed an equivalent or superior killing activity in 2 out of 3 ALL lines tested. We are currently testing the in vivo activity of anti-CD22 CAR in an immunodeficient mouse model bearing human cell lines and primary patient-derived ALL. Our results indicate that CD22-CAR should be developed for the immunotherapy of CD22+ hematologic malignancies.

Key Words: Adoptive therapy, CD8+ T cells, Leukemia.

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COSTIMULATION BY CD27, NOT CD28, SIGNIFICANTLY AUGMENTS THE SURVIVAL OF GENETICALLY REDIRECTED HUMAN T CELLS IN VIVO

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The costimulatory effects of CD27 on T lymphocyte effector function and memory formation has been confined to evaluations in mouse models, in vitro human cell culture systems and observations from the clinic. Whether CD27 costimulation actively enhances human T cell function, expansion and survival in vitro and in vivo remains unclear. Primary human T cells that were engineered to express an antigen-specific chimeric antigen receptor (CAR)

containing an intracellular T cell receptor CD3 zeta (CD3-z) chain signaling module with the CD27 costimulatory motif in tandem exerted increased effector functions in vitro, including antigen-stimulated Th-1 cytokine secretion and cytotoxicity, compared to CAR T cells with CD3-z signaling alone. After stimulation in vitro, CAR T cells harboring the CD27 domain also proliferated, upregulated Bcl-XL protein expression, resisted antigen-induced apoptosis and underwent increased numerical expansion. However, the greatest impact of CD27 costimulation was noted in vivo where transferred human T cells expressing the CD27 costimulated CAR demonstrated heightened persistence, which exceeded that of a CAR of similar specificity which contained a CD28 endodomain. CD27 costimulation facilitated improved regression of large, established human cancer in a xenogeneic allograft model. Thus, CD27 costimulation enhances the expansion, effector function and survival of human CAR-expressing T cells in vitro and augments human T cell persistence and anti-tumor activity in vivo. Additionally developing results will be presented at the meeting.

Key Words: Adoptive therapy, Ovarian cancer, Tumor associated antigen.

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NEW GMP-GRADE, XENO-COMPONENT FREE MEDIUM FOR THE ACTIVATION AND EXPANSION OF T CELLS

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Therapeutic applications of T cells in immunotherapy have recently gained momentum with the promising results in adoptive transfer of antigen-specific T cells for infectious complications after allogeneic stem cell or solid organ transplantation or for immunotherapy of malignant diseases. Activation and expansion of these cells for clinical application under controlled conditions requires GMP-grade reagents including appropriate antibodies, cytokines and media. For these standardized, reproducible cell cultivation procedures and ex vivo differentiation, a new xeno- and serum-free, GMP-grade medium (TexMACS GMP Medium) for clinical use has been developed. The expansion of T cells in TexMACS GMP Medium upon polyclonal activation using biotinylated antibodies against CD2/ CD3/ CD28 loaded on anti-Biotin-MACSiBeads, resulted in expansion rates comparable to what was observed with other commercially available serum-free media. T cell expansion in TexMACS GMP Medium after activation with CD3 and CD28 soluble antibodies in high density systems (e.g. Wilson Wolf®) showed high expansion rates of viable and functional T cells at densities of more than 5x107 cells/ mL. The generation of antigen-specific T cells with the CliniMACS Cytokine Capture System (IFN-gamma) using the TexMACS GMP Medium showed comparable results regarding purity and recovery to the use of RPMI + 10% human AB serum. For the automation of such complex procedures, a new cell processing device (CliniMACS Prodigy) was developed. All steps in antigen-specific T cell processing, i.e. antigen-specific re-stimulation, magnetic enrichment, and in vitro expansion in TexMACS GMP Medium are performed in this fully automated device, in a closed system under sterile conditions. In conclusion, the developed GMP-grade and xeno-component free TexMACS GMP Medium showed comparable or superior performance to other commercially available serum-free media in all tested T cell activation and expansion systems. The medium and other relevant cell culture components (e.g. antigens, antibodies, cytokines etc.) are harmonized with all components of the CliniMACS® system enabling clinicians to pursue contemporary clinical bench to bedside research.

Key Words: CD4+ T cells, CD8+ T cells, Cytokine.

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RHAMM/HMMR-SPECIFIC LYMPHOCYTES EFFICIENTLY LIMIT TUMOR OUTGROWTH IN VIVO

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Rhamm/HMMR (hyaloronan-mediated motility receptor) is a well-characterized leukemia-associated antigen that is over-expressed in leukemic cells and several other tumor entities, including melanoma and breast cancer. Clinical studies using an HMMR-derived peptide vaccination in acute myeloid leukemia (AML) patients resulted in encouraging clinical benefit and detection of HMMR-specific cytotoxic T cells has been associated with regression of disease. Therefore, we selected HMMR as a potential target antigen for adoptive T cell therapy. Allo-restricted high-avidity T cell clones were isolated and TCR genes were transferred into recipient lymphocytes and shown to recognize tumor cells in vitro.

To address the in vivo antitumor effect of TCR-transgenic lymphocytes in a xenograft mouse model we choose one Rhamm/HMMR-specific TCR (TCR150), which showed tumor-specificity in in vitro analysis. First, a solid tumor model was established using subcutaneous inoculation of a human melanoma cell line (HLA-A2+, HMMR+) into NOD/scid IL2Rgnull (NSG) mice. Second, a luciferase-tagged AML cell line was inoculated intravenously into NSG mice, enabling us to investigate the potential of the TCR150-expressing lymphocytes to retard human disseminated tumor cells. Adoptive transfer experiments indicated that a cell dose of only $2x10^5$ lymphocytes expressing TCR150 was sufficient to significantly limit tumor outgrowth. As the phenotype of recipient cells is also important for antitumor functionality in vivo, the use of memory phenotypes was examined. Various culture conditions, using IL-7 or IL-15, resulted in a central memory (TCM) or effector memory (TEM) phenotypes, respectively. Transfer of TEM and TCM lymphocyte populations into tumor-bearing mice resulted in an enhanced tumor retardation compared to conventionally prepared, IL-2-treated lymphocytes. This effect became even more prominent when exogenous IL-15 was administered, prolonging

the lymphocyte survival. Moreover, the most profound limitation of tumor outgrowth was observed when exogenous IL-15-treatment was combined with the administration of CD8-enriched TCR-transgenic lymphocytes.

Key Words: Adoptive therapy, Animal model, CD8+ T cells.

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A UNIVERSAL IMMUNE RECEPTOR EXPRESSED BY T CELLS FOR THE TARGETING OF DIVERSE AND MULTIPLE TUMOR ASSOCIATED ANTIGENS

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Adoptive Cell Transfer (ACT) of T cells transduced to express a chimeric antigen receptor (CAR) is an attractive and promising treatment for human malignancies. CARs have fixed antigen specificity most commonly provided by a fused antibody single-chain fragment, which allows for exclusive targeting to only one tumor associated antigen (TAA). One CAR may have limited efficacy in the clinic, since tumors are highly heterogeneous and antigen expression differs markedly between tumors among individuals, and even within the same patient. To ensure widespread efficacy of CAR therapy it would be necessary to produce a large and costly panel of CARs against an array of known TAAs, to be tailored for each individual. We therefore sought extend the recognition specificity potential of bioengineered T lymphocytes by developing a novel, universal and versatile strategy that allows flexibility in redirecting T cells against multiple TAA. Here, we designed a biotin-binding immune receptor (BBIR) which encodes an extracellular modified avidin in pseudo- dimeric form linked to an intracellular T cell signaling domain. BBIR T cells recognize and bind exclusively to biotinylated molecules, including scFvs, antibodies or other reagents, that specifically bind to cancer cells according to the array of known antigens expressed on the tumor membrane. In plate bound assays, where immobilized tumor antigen is secondarily bound by biotinylated reagents, binding of the BBIR to biotin triggers antigen-specific T cell effector activity. Further, binding of biotinylated antibodies to mesothelin, folate binding protein or EpCAM TAAs on the tumor cell surface allows BBIR T cells to recognize various tumor cell lines with non-overlapping antigen expression and triggers their secretion of various proinflamatory cytokines and cytotoxic activity in vitro. The versatility afforded by the BBIR platform allows for both sequential and simultaneous targeting of a combination of distinct antigens expressed by tumor. Moreover, BBIR T cells possess antitumor activity in vivo with significant inhibition of tumor outgrowth in co-administration assays and against established tumor in immunodeficient mice. In conclusion, we report the development of a novel system of versatile T cell specificity, the biotin binding immune receptor, which extends beyond conventional CAR approaches for the tailored generation of T cells of unlimited antigen-specificity.

Key Words: Adoptive therapy, Chimeric receptors, Ovarian cancer.

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RETARGETED NATURAL KILLER CELLS FOR ADOPTIVE CANCER IMMUNOTHERAPY

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Natural killer (NK) cells are the body's first line of defense against viral infections and malignant cells. In addition to autologous or donor-derived primary NK cells, also continuously growing cytotoxic cell lines such as NK-92 hold promise for cancer immunotherapy. In preclinical studies NK-92 showed high cytotoxicity against malignant cells of hematologic origin, and safety of infusion of high doses of NK-92 cells was established in phase I clinical trials utilizing irradiated cells to prevent permanent engraftment. To further enhance their therapeutic utility and provide NK-92 cells with predetermined tumor-cell specificity, we previously generated variants genetically modified with retroviral vectors to express chimeric antigen receptors (CAR). These prototypic CAR were composed of a tumor-specific single chain Fv antibody fragment fused via a flexible hinge region to CD3 zeta chain as an intracellular signaling moiety. The retargeted NK cells displayed high cytotoxic activity, and selectively killed antigen positive and otherwise NK-resistant targets. As a prerequisite for application of ErbB2-specific NK cells in cancer patients, here we developed novel CAR constructs that were humanized to reduce potential immunogenicity, and codon optimized to improve expression in NK-92 cells. In addition to an optimized CAR employing the ErbB2-specific scFv(FRP5) antibody fragment for target-cell recognition and human CD3 zeta chain as a signaling moiety, we also investigated CAR with composite signaling domains. CAR constructs were stably introduced into NK-92 by lentiviral gene transfer, CAR-expressing single cell clones were isolated by FACS sorting, and functionality was characterized. Thereby CAR containing CD3 zeta (5.z), or CD28 and CD3 zeta domains (5.28.z) showed highest expression levels, with NK cells expressing the latter displaying most selective killing activity towards ErbB2-expressing breast carcinoma, medulloblastoma and glioblastoma cells. Based on these results, a clinically applicable lentiviral 5.28.z CAR vector was derived, and protocols for GMP-compliant transduction of NK-92 cells were developed. Ongoing work now focuses on molecular and functional characterization of the resulting NK-92/5.28.z cells to identify clonal cell lines most suitable for clinical development.

Key Words: Adoptive therapy, Chimeric receptors, NK cells.

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T CELL RECEPTOR DERIVED FROM AUTOIMMUNE VITILIGO T CELLS SHOW HIGH AFFINITY AGAINST MELANOMA

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A gene therapy approach where retroviral vectors encoding the T cell receptor (TCR) genes from tumor or reactive T cell clones have been used to transfer the anti-viral reactivity to normal PBL-derived T cells. The clinical responses have been observed in these trials. However these clinical responses still remain low. One major problem may be the affinity of TCR is not sufficiently high. Vitiligo in healthy individuals is thought to be related to the autoimmune response directed against melanocytes. We have cloned and expressed three TCRs targeting a HLA-A2 restricted tyrosinase antigen by tetramer staining from the healthy individuals with vitiligos. Our in vitro results have shown that either CD8+ T cells or CD4+ T cells that have been genetically modified with the TCR genes would recognize the melanoma cells which express the tyrosinase antigen, indicating the TCRs have the higher affinities. We propose here that this may be an alternative strategy to obtain high-affinify TCRs for the treatment of the patients with melanoma.

Key Words: Adoptive therapy, CD4+ T cells, CD8+ T cells.

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IMMUNOTRANSPLANT FOR MANTLE CELL LYMPHOMA: A PHASE I/II STUDY DEMONSTRATING AMPLIFICATION OF TUMOR-REACTIVE T CELLS

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Mantle cell lymphoma (MCL) has a poor prognosis. Though autologous transplant prolongs survival, novel therapies are needed to target residual, myeloablation-resistant tumor cells that result in relapse.

Trials of CpG-based vaccines for low-grade lymphoma have shown induction of anti-tumor T cells and clinical responses [Brody et al, JCO 2010]. In a pre-clinical model, we developed 'immunotransplant', combining: 1) CpG-based vaccination, 2) vaccine-primed T cell harvest, 3) myeloablation with stem cell rescue, and 4) T cell re-infusion. Immunotransplant amplifies the proportion of anti-tumor T cells by an order of magnitude and cures even bulky, systemic lymphoma [Brody et al, Blood 2009].

METHODS: We initiated a phase I/II study of immunotransplant for newly diagnosed MCL patients to test the hypothesis that immunotransplant amplifies anti-tumor T cells as seen pre-clinically. Tumor-reactive T cells are assessed by co-culturing autologous tumor with peripheral blood T cells and measuring their production of: IFNγ, TNF, IL2, CD137, perforin and granzyme by multiplex surface and intracellular flow cytometry. Using high-throughput sequencing, we have initiated assessment of the peripheral blood TCRβ repertoire, obtaining at least 1e6 sequence reads per time point.

RESULTS: Accrual has been rapid with 24 patients enrolled in 22 months and 13 patients completing the complete protocol so far. Flow-cytometric immune response testing has demonstrated that immunotransplant amplifies the proportion of tumor-reactive T cells in 83% of patients thus far. Notably, we have observed some patients with primarily CD8 T cell responses, some with CD4 T cell responses, and some with a combination. In some cases, tumor-reactive T cells have been tested for reactivity to autologous, non-malignant B cells and have demonstrated that a significant proportion are tumor-specific. High throughput sequencing of TCRβ repertoires have also demonstrated instances of significant clonal amplification after immunotransplantation, up to two orders of magnitude. In extreme cases, these have yielded dominant clones comprising as much as 50% of a patient's entire peripheral blood T cell repertoire post-transplant.

CONCLUSIONS: Pre-clinically, amplification of anti-tumor T cells correlates with cure of myeloablation-resistant disease. The reiteration of anti-tumor T cell amplification in our preliminary clinical data raises the possibility that immunotransplant may improve clinical outcomes. Our ongoing molecular residual disease testing should suggest whether certain patterns of T cell response correlate with clinical benefit and whether the cohort has a better-than-expected molecular remission rate.

Key Words: Adoptive therapy, Cancer vaccine, Lymphoma.

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DISSECTION OF HUMAN MELANOMA-SPECIFIC T-CELL IMMUNITY

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Tumor-infiltrating lymphocytes isolated from patients with metastatic melanoma and expanded in vitro using high-dose IL-2 have been used for adoptive transfer with impressive clinical results; however, the antigen specificities of the T cells in these cellular products have not been determined. Using a compilation of all described melanoma-associated antigens and a recently developed technology for high-throughput analysis of T-cell responses, we dissected the composition of melanoma-restricted T-cell responses in tumor-infiltrating lymphocyte cultures. Using this platform, we screened 63 tumor-infiltrating lymphocyte cultures for T cell reactivity against 175 melanoma-associated epitopes and detected in total 90 responses against 18 different epitopes—predominantly from differentiation and cancer-testis antigen origin. Notably, the majority of these responses were of low frequency and tumor-specific T-cell frequencies decreased during rapid expansion. Furthermore, we observed a large variation in the T-cell specificities detected in cultures established from different fragments of resected melanoma lesions. Finally, recognition of autologous tumor versus peptide recognition, as described by our peptide library, suggested that the low-frequency tumor-restricted T-cell populations found in tumor-infiltrating lymphocytes are responsible for a considerable part of the HLA-A2 restricted tumor recognition.

In addition to the valuable insight in the composition of TIL cultures used for adoptive transfer, this screening proofs the ability to perform T cell profiling to a large array of T-cell epitopes that can be used to assess the effects of the large number of immuno-active compounds that is currently in clinical development, and data set provided here should provide a benchmark in such studies.

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

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DETECTION OF ANTIGEN-STIMULATED CYTOKINE-SECRETION IN T-CELLS AT SINGLE CELL LEVELS ON A LIVE CELL CHIP

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Previously we established "on chip single cell analysis method" called ISAAC (immune-spot array assay on a chip), which enabled us to detect secretion of antigen-specific antibody (Ab) from single Ab-producing cells and which provided us very efficient and rapid method to produce antigen-specific Ab from any animal species. In this study, we applied the ISAAC to characterize T-cells. To this end, we used two lines of transgenic mice whose transgene encoded TCR recognizing male-specific H-Y peptide presented on H-2Db (H-Y TCR) or that encoded TCR recognizing ovalbumin-derived peptide presented on H-2Kb (OT-1 TCR). We used microwell array chips that have an array of 45,000 to 234,000 microwells whose shape and size are just fit to accomodate single lymphocytes. By applying lymphocytes onto the chip whose surface was coated with anti-cytokine Ab, we could prepare an array of single lymphocytes, stimulate cells on the chip and detect cytokine-secretion from single lymphocytes on the chip. We could detect secreted cytokines using fluorophore-labeled anti-cytokine Ab as doughnut-like spots that enabled us to easily distinguish signals and noises. To stimulate cells on the chip we used MHC/peptide tetramers. When we stimulated T-cells derived from H-Y TCR transgenic mice on the chip with H-Y tetramers, we observed IL-2-secretion from single lymphocytes on the chip, but not with OT-1 tetramers. In contrast, we observed IL-2-secretion from lymphocytes of OT-1 TCR transgenic mice on the chip by stimulating cells with OT-1 tetramers but not with H-Y tetramers. The results show that on chip method enables us to detect antigen-specific T-cells with high specificity. We named the method T-cell ISAAC. T-cell ISAAC might provide the very efficient and rapid detection method of antigen-specific T-cells in primary T-cell population and facilitate the antigen-specific T-cell therapy in the future.

Key Words: CD8+ T cells, Cytokine.

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DIRECT TCR EXPRESSION ASSAY (DTEA) FOR QUANTIFYING T-CELL RECEPTOR α-CHAIN AND β-CHAIN DIVERSITY USING THE DIGITAL NCOUNTER™ ASSAY SYSTEM AND ITS IMPLICATIONS IN ADOPTIVE T CELL THERAPY

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Measurement and skewing of T-cell receptor (TCR) α and β diversity in the tumor-bearing host (i) engrafted with allogeneic hematopoietic stem cells and/or (ii) after vaccination or adoptive T-cell transfer, are used for monitoring the effect of immunotherapies. TCR diversity is mostly concentrated within the third complementarity-determining region (CDR3) of α and β chains. Currently available methods for measuring diversity are primarily limited to TCRVβ usage and are (i) flow-cytometric detection by using antibody panel against TCRVβ, (ii) CDR3 length spectratyping by semi-quantitative RT-PCR, and (iii) DNA sequencing of the rearranged CDR3 regions. Although these methods can detect oligoclonal expansion of T cells they are costly, labor-intensive, and require substantial amount of sample. Herein, we adapted the newly-developed Nanostring nCounter™ Analysis System to directly quantify gene expression of 46 TCRVβ and 45 TCRVα gene families in a single non-enzymatic reaction (DTEA) from a small number of T cells and or very minimal amount of total RNA. Two sequence-specific gene probes, one mRNA target sequence-specific capture probe, and a second mRNA target sequence-specific fluorescent-labeled color-coded probe were produced for each of the TCR genes. These were used to determine TCRVα and TCRVβ usage based on profiling the 99 V region families of T cells from healthy volunteers by the nCounter™ System and a subset of these data were validated by flow cytometry with TCRVβ-specific mAb. Subsequently, we analyzed TILs and peripheral blood RNA samples obtained from patients with advanced melanoma treated with ex vivo propagated tumor infiltrating lymphocyte (TIL) therapy. Compared with sequencing the CDR3 of TCR, DTEA yielded similar results in detecting major Vα and Vβ genes but was more rapid and sensitive and was able to simultaneously track the full array of Vα and Vβ genes in each sample. In patients undergoing adoptive T-cell therapy, DTEA can be used to serially track TCR gene usage when the identities of CDR3 sequences are not required. This is the first time that digital mRNA profiling for TCRVβ and TCRVα has been undertaken without requiring cDNA synthesis, enzymatic reactions, or mAb usage. Our approach requires just a small amount of total RNA and is cost-effective. It is anticipated that this approach will be of significant interest to immunologists seeking to manipulate the human immune system.

Key Words: Adoptive therapy, CD4+ T cells, CD8+ T cells.

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PREDICTION OF CANCER IMMUNOTHERAPY RESPONDERS BY QUANTIFYING EX VIVO INDUCED MESSENGER RNAs IN WHOLE BLOOD

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Although recent progress of cancer immunotherapy is demonstrating a lot of hope, it is not beneficial for every patient, and we do not know who respond to the therapy, or not. If such responders are predicted with a high level of confidence, immunotherapy may be the treatment choice, even though it is experimental and expensive. Since immune function is very complex, and various components are interacting with each other, new diagnostic methodology is needed. Using the method we developed recently (J Immunol Methods 363:95-100, 2010), we quantified 17 different leukocyte-function-associated mRNAs (ACTB, B2M, IFNG, TNFSF1, 2, and 5, IL2, 8, and 10, TGFB, CTLA4, PDCD1, FOXP3, GMCSF, VEGF, CCL8, and CXCL3) in heparinized whole blood after ***ex vivo*** stimulation with 8 different agents (phytohemagglutinin-L (PHA), heat aggregated IgG, zymosan, recombinant human IL2 (rIL2) and IFNα2b, mouse monoclonal antibody against α/β chain of T cell receptor, picibanil, and phosphate buffered saline. ***Ex vivo*** stimulation was carried out at 37°C for only 4 hours using heparinized whole blood obtained from patients before dendritic cell vaccine therapy. Blood volume needed for this assay was as small as 1.5 mL. We recruited 26 advanced cancer patients with a variety of cancer types, and the clinical outcome (PD, SD, and PR) was determined by the RECIST criteria, without knowing mRNA data. The fold increase of the control genes (ACTB and B2M) was not different among the three groups, suggesting that the assay was performed appropriately. Zymosan-induced IFNG and IL10 were significantly higher in PR than SD ($p=0.04$) or PD and SD combined (IFNG, $p=0.02$). In contrast, PHA- and rIL2-induced TNFSF2 were significantly ($p=0.006$ for PHA, $p=0.04$ for rIL2) higher in PD than PR or SD and PR combined. PHA-induced IL10 in SD was significantly lower than PD ($p=0.04$) or PR ($p=0.02$), respectively. Even though statistically significant changes were identified, individual mRNA data were not applicable for diagnostic testing due to the large overlap of values among PD, SD, and PR. However, PD, SD, and PR were predicted 100% by multivariate discriminant analysis using the combination of at least three different mRNAs or at least two distinct stimulations. The parameter of each stimulant-mRNA combination generated by multivariate analysis was used to create the formula for the prediction of PD, SD, and PR. Since this method is technically applicable to various clinical sites, it will be a model of personalized medicine diagnostics for cancer immunotherapy. This method will also be an attractive tool for clinical trials of cancer immunotherapy.

Key Words: Cellular immunity, Cytokine, Dendritic cell.

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EVALUATION OF ISOTOPIC AND NON-ISOTOPIC TECHNOLOGIES FOR ASSESSING POTENCY OF CHIMERIC FUSION IMMUNOTOXINS CONSISTING OF IL-13 OR IL-4 AND TRUNCATED PSEUDOMONAS EXOTOXIN IN RECEPTOR POSITIVE HUMAN CANCER CELLS

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Previously, we have demonstrated that a variety of human tumors and tumor cell lines overexpress high density receptors for Th2 derived cytokines, IL-13 and IL-4. Because IL-13Rα2 and IL-4Rα chains are primary binding components of the IL-13R and IL-4R system, respectively and they are targets for IL-13PE and IL-4PE immunotoxins, there is a need to develop novel potency assays to test the characteristics of these and other immunotoxins and agents. In the present study, we developed and tested isotopic and non-isotopic assays for measuring biological activity of these two immunotoxins using fluorogenic and chromogenic assays, which measure the release of certain metabolites or DNA from viable cells after incubation with immunotoxins. We compared the data with isotopic techniques that utilize 3H-leucine incorporation and 3H-thymidine uptake. The end-point for flurogenic assay is based on reduction of the fluorogenic chromogen, resazurin, which excites at 544nm and emits at 590nm wavelength by cytosolic dehyrogenases released by dying tumor cells. The read-out for MTS assay is based on increase in the chromogenic intensity at 490 nm wavelength. The fluorogenic (resazurin) and chromogenic (MTS) techniques in three high IL-13R overexpressing and one low IL-13R expressing human tumor cell lines showed similar profile of cytotoxicity. The IC50 (the concentration of the immunotoxin that kills 50% of the tumor cells) obtained by non-isotopic assays positively corroborated with the isotopic assay (3H-Leucine and 3H-thymidine uptake). Interestingly, the end-point read out of the resazurin reduction is achieved within the first four hours of the assay compared to overnight incubation with 3H-thymidine or 4 hours with 3H-leucine incorporation. In addition, these techniques eliminated three lengthy steps involved in isotopic techniques such as freezing and thawing cells, cell harvesting, and drying of filter-mats before counting 3H-leucine or 3H-thymidine incorporation. In addition, these assays did not generate any radio active waste. Our results indicate that non-isotopic assays represent a useful alternative approach for screening potency of these immunotoxins for translational research. These assays may also be applicable for testing cytotoxicity of other agents.

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

Presenting author underlined. *Priminary author in italics.*

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MODULATION OF IMMUNE CELL SUBPOPULATIONS IN RENAL CELL CARCINOMA PATIENTS BY SUNITINIB

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Background: Increased numbers of immune suppressive cells, like regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) and alterations in the frequency and function of activated and memory B and T cells and NK cells in peripheral blood mononuclear cells (PBMNC) represent immune escape mechanisms of renal cell carcinoma (RCC). The tyrosine kinase inhibitor sunitinib, which is currently successfully implemented in the treatment of RCC, has been shown to modulate immune suppressive and immune stimulatory thereby shifting the immune balance in these patients to a more stimulating setting.

Methods: Using multicolour flow cytometry and a large panel of antibodies directed against different immunomodulatory molecules, we here analysed the effects of (i) adjuvant sunitinib treatment (50 mg for 4 weeks, 2 weeks off) on the frequency and composition of immune cell populations, their function and activity in 10 RCC patients and (ii) sunitinib treatment of PBMNC from healthy donors over time

Results: A time-dependent decrease in the frequency of Treg was found in PBMNCs during in vitro and in vivo sunitinib treatment in particular in sunitinib responders, while the number of Tregs was not or only marginally reduced in non-responders. In addition, the number of NK cells, activated and/or memory T cells was increased in PBMNC of TKI-sensitive patients with a complete or partial response over time, whereas it remained unaltered in patients with only minor responses or resistance.

Conclusions: This observation may have important implications for (i) the monitoring of the sunitinib activity on immune cells, (ii) the treatment schedule including duration of TKI therapy and (iii) the understanding of the development of sunitinib resistance. Long-term monitoring of a larger patients' group and association with clinical parameters will show whether the activation of T cells and NK cells as well as the decrease of immune suppressive cells due to sunitinib treatment is associated with enhanced anti-tumor responses and TKI resistance/sensitivity.

Key Words: Cellular immunity, Renal cell carcinoma, Targeted therapeutics.

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CHANGES IN ICOS+ AND ICOS- POPULATIONS OF MEMORY AND REGULATORY T CELLS IN STAGE IV MELANOMA PATIENTS UNDERGOING ANTI-CTLA4 THERAPY

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In this work we compare ICOS+ and ICOS- populations of memory T cells and regulatory T cells (Tregs) in stage IV melanoma patients (n=12) undergoing anti-CTLA4 therapy. PBMCs were collected at baseline, week 7, and week 12. One 12-color flow cytometry panel characterized activation markers (ICOS, Ki67, PD1, NKG2D, and CTLA4) on memory phenotypes (CD3, CD4, CD8, CCR7, CD45RA, CD27, and CD28). Another 10-color panel characterized activation markers (ICOS, CD45RA, and Helios) on CD4+ Tregs (CD3, CD4, CD25, and FoxP3). This panel also included CTLA4, PD1, and OX40, but none of the patients showed any appreciable expression of these markers on Tregs. Using Exhaustive Expansion (Siebert et al., Journal of Translational Medicine, 2010), we looked for patterns of expansion and contraction in nearly 40,000 subsets from the memory/effector panel and 243 subsets from the Treg panel. We observed inter-patient heterogeneity with regard to increased expression of activation markers (CTLA4, NKG2D, and PD1) on memory/effector subphenotypes. In conclusion, multi-parameter flow cytometry, coupled with appropriate computational approaches, offers a powerful platform for high-throughput immune monitoring at a single cell level. We are able to characterize multiple patterns of activation on multiple phenotypes, and to track longitudinal changes in these patterns during immunotherapy.

Key Words: Active immunotherapy, CTLA-4, Treg cells.

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A NOVEL COLORECTAL CANCER VACCINE CONSISTING OF MULTIPLE NATURALLY PRESENTED PEPTIDES

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To effectively treat cancer patients with T cell-based immunotherapy, T cells have to recognize peptides restricted by HLA molecules on tumors. For most of the published peptides it is unknown whether they are naturally presented or not.

IMA910 is a novel peptide-based vaccine consisting of 10 HLA-A*02 binding and 3 HLA-DR binding tumor associated peptides (TUMAP), which were presented on colorectal tumors and were overexpressed in comparison to healthy tissues. 8 of the 10 A*02-binding peptides had been confirmed to be naturally presented by peptide elution from surgically resected tumors and analysis by high-sensitivity mass spectrometry (XPRESIDENT approach). The two other A*02-binding peptides were chosen due to their characteristics described in literature.

The multi-center clinical trial IMA910-101 enrolled 92 HLA-A*02+ advanced colorectal cancer (CRC) patients being at least clinically stable after 12 weeks of first-line oxaliplatin-based therapy. Patients were infused with a single low dose of cyclophosphamide (300mg/m2) and repeatedly immunized intradermally (up to 16 vaccinations) with IMA910 in combination with GM-CSF (cohort 1; n=66) or IMA910 with GM-CSF plus topically applied imiquimod (cohort 2; n=26) as immunomodulators. Before and post vaccination patients were analyzed for T-cell responses to IMA910 HLA-A*02 and HLA-DR restricted peptides by HLA-multimer assay and intra-cellular cytokine (ICS) assay for CD8 T-cell responses and by ICS assay for CD4 T-cell responses. Tumor status of patients was monitored repeatedly by CT/ MRI according to RECIST, corresponding tumor scans were reviewed centrally for assessment of disease control rate (DCR), progression-free survival (PFS) and overall survival (OS).

IMA910 overall was immunogenic in 75/80 (94%) evaluable patients. A moderate but significant effect of imiquimod treatment on the number of immune responses to IMA910 peptides as detected by the ICS assay was observed. Finally, we demonstrate significantly increased PFS and a trend for increased OS in patients with class I immune responses to multiple TUMAPs. Most interestingly, only immune responses to peptides confirmed to be naturally presented were significantly associated with OS. Responses to the two immunogenic peptides included from literature with unclear status of natural presentation did not correlate and actually diluted the overall correlation of immune response with clinical benefit. This latter finding suggests that peptide antigens confirmed to be naturally presented may be preferable for vaccination and immunomonitoring.

Key Words: Active immunotherapy, cancer vaccine, colorectal cancer.

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THE GENETICS DRIVING TH1 VS TH17 IMMUNE RESPONSES; A COMPARATIVE ANALYSIS OF MELANOMA METASTASES AND DESCENDENT CELL LINES

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The weight that alterations in genomic copy number play in oncogenesis and in the determination of global transcriptional patterns remains controversial. Although individual gene expression has been linked to copy number variation, such relationship cannot be directly extrapolated at the whole genome level. Here, we hypothesized that transcripts stably expressed in melanoma cell lines (CMs) in vitro compared to their parental tissue (TMs) in vivo may be more closely related to oncogenesis. We observed that the higher the stability of expression between CMs and TMs, the higher the likelihood that the abundance of a given gene was concordant to its predicted transcription according to copy number analysis. Functional interpretation of 3,030 transcripts similarly (Pearson's p-value < 0.05) expressed by CMs and TMs confirmed that most were related to oncogenesis. Among them, 968 were expressed in concordance with the transcriptional efficiency predicted by copy number analysis. We named this subset of genes, genomic delegates and used them to test whether they could segregate a broad array of melanoma metastases. This exercise identified two distinct subpopulations of melanoma one characterized by high expression of cancer testing antigens and a Th17 immune phenotype (Group A) and a second characterized by high frequency of expression of melanoma differentiation antigens and a Th1 type of immune environment (Group B). A third category could be indentified that set in between the two polar groups (Group C). The three groups could be confirmed by whole genome transcriptional comparison by principal component analysis which clearly demonstrated a natural separation accordingly to the prediction based on delegate genes. Moreover, the same separation could be observed using as a data base known oncogenes or tumor suppressors associated with melanoma; in that case, Group A was enriched of transcripts related to cyclins, WNT and g-protein coupled receptor signaling while Group B was enriched with transcripts related to MAP kinase pathways and canonical melanoma signaling.

Key Words: Melanoma, Th1/Th2 polarization, Tumor associated antigen.

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IN VIVO IMAGING FOR ASSESSING BIODISTRIBUTION OF IL-13PE IMMUNOTOXIN IN A MOUSE MODEL OF GLIOBLASTOMA

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Objectives: Since Interleukin-13 Pseudomonas exotoxin (IL-13PE) is a powerful cytotoxic agent against IL-13 receptor α2 (IL-13Rα2)-expressing tumors, it is being tested for the treatment for glioblastoma multiforme (GBM). However, results using convection-enhanced delivery (CED) of IL-13PE have been disappointing in part because it is difficult to track the distribution of the drug with respect to the tumor. To evaluate the biodistribution of IL-13PE in a mouse model of GBM, we radiolabeled IL-13PE with ^{125}I, stereotactically injected it and imaged the brain with a micro-SPECT/CT. We also conjugated IL-13PE with a near-infrared (NIR) fluorochrome 780 to assess its distribution using an ***in vivo*** imaging system.

Methods: IL-13PE was labeled with either ^{125}I or NIR fluorochrome and evaluated its binding and functional activity in IL-13Rα2 expressing U251 human glioblastoma or PMRCC renal cell carcinoma cell lines. T98G, an IL-13Rα2 negative glioma cell line, served as a negative control. Nude mice with intracranially implanted human glioblastoma tumors were given stereotactic intratumoral injections of ^{125}I-IL-13PE followed by SPECT/CT imaging.

Results: Both ^{125}I-IL-13PE and NIR-IL-13PE demonstrated highly specific binding to IL-13Rα2 positive tumor cells and were cytotoxic to both PM-RCC and U251 but not to T98G cells. Both the binding and cytotoxic activities were blocked by a 100-fold excess of IL-13, which indicated that IL-13PE binding was specific. Next, U251 cells were stereotactically implanted in mice brains and a bolus dose of ^{125}I-IL-13PE was administered intracranially. SPECT/CT imaging revealed retention of ^{125}I-IL-13PE in mouse brain tumors.

Conclusions: CED is a loco-regional drug delivery method for brain tumors that relies on a continuous pressure gradient to distribute drug into interstitial space. We are working on SPECT/CT imaging after CED of ^{125}I-IL-13PE to evaluate intracranial distribution of the immunotoxin. Similarly, our ongoing experiments on NIR-IL-13PE with a fluorescence ***in vivo*** imaging will confirm the SPECT/CT results to image biodistribution of IL-13PE. Thus, we have developed a novel technology to label IL-13PE for ***in vivo*** imaging without loss of its binding or cytotoxic activity. ^{125}I-IL-13PE appears to be useful for SPECT imaging. We are currently evaluating critical parameters such as activity, distribution and toxicity of ^{125}I-IL-13PE and NIR-IL-13PE in mouse brain to improve IL-13PE delivery for use as an effective therapy for intracranial tumors.

Key Words: Animal model, Cytokine, Tumor associated antigen.

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ARGINASE 2 AND INOS EXPRESSION AS POTENTIAL PROGNOSTIC AND PREDICTIVE BIOMARKERS OF SURVIVAL IN RENAL CELL CARCINOMA

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There is a great deal of variability in the survival of patients diagnosed with renal cell carcinoma (RCC) that is not explained by the classical clinical measures of cancer aggressiveness such as tumor stage or grade. The latter indicators are surrogate measures of what is actually happening at the metabolic and molecular level within the tumor itself. Finding metabolic and molecular signatures in RCC tumors could lead us to identify the risk of cancer progression and to determine patient survival. These facts will improve our ability to predict clinical outcomes, direct patient therapy and determine new targets for drug development. To date, most of the biomarker research in RCC has centered on bio-products of the von Hippel-Lindau (VHL) pathway including VHL mutations, VEGF, and hypoxia-inducible factor. We have shown that the enzymes arginase 2 (Arg2), inducible nitric oxide synthase (iNOS) and their metabolic products, can play an important role in tumor growth and tumor suppression. It appears that an increase in Arg2 activity correlates with the need for malignant cells to use polyamines at a high rate to sustain rapid proliferation, reducing significantly the local and systemic levels of L-Arginine. In contrast, iNOS and nitric oxide (NO) functions are related to tumor growth, cellular differentiation and formation of metastasis when expressed at low levels. Arg2 has been shown to be increased in breast, colon and prostate cancer. We have found by immunohistochemistry that Arg2 is expressed at high levels in high grade clear RCC tumors compared to those presenting with low grade of differentiation. In addition, the high Arg2 expressing tumors express low levels of iNOS when compared to normal kidney controls. Protein expression of the two enzymes and their metabolic products were confirmed by enzymatic assays, western blots and High Performance Liquid Chromatography respectively. The differences in high Arg2 and low iNOS expression appear to correlate ($r=0.913$) with the progression of the disease and short free survival time in contrast to those tumors exhibiting low Arg2 and high iNOS expression. The results of this study will result in a clear and better understanding of the role that Arg2, iNOS and their metabolites play in the development and etiology of RCC. The knowledge of these metabolic biomarkers will have a positive and significant impact on RCC patient care by more accurately identifying individuals that will directly benefit from any giving treatment. Furthermore, these biomarkers can provide sensitive indicators of tumor activity and response to treatment.

Key Words: Renal cell carcinoma, Targeted therapeutics, Tumor microenvironment.

Presenting author underlined. *Priminary author in italics.*

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VALIDATION OF ELISPOT ASSAYS IN 384 WELL FORMAT - 400 TESTS DONE WITH 10 ML BLOOD

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The amount of blood that is obtainable for cellular assays is limited, yet, the number of immune parameters to be tested ideally is steadily increasing. Since determinant spreading regularly occurs in anti-tumor immunity, it is wise to test for several tumor antigens and for several peptides of each antigen. Titrating each peptide is advisable since it provides information on the avidity of the T cell response. Furthermore,by measuring different analytes, such as, IFN-γ, IL-17, IL-2, IL-4, Granzyme B, etc, one can define the effector T cell lineage that has been engaged. ELISPOT assays are already highly efficient in cell utilization, because 100,000 PBMCs suffice for each data point when measured in the standard 96 well plate format. Moreover, the cells can be recycled and tested in several subsequent ELISPOT assays, further increasing the efficacy of cell utilization. In our presentation, we will focus on serial testing of PBMC. In addition, we will introduce ELISPOT assays in 384 well format, which enables analysis with as few as 25,000 PBMC per test. Therefore, using the 384 well format, up to 400 ELISPOT tests can be performed with 10 million PBMC, normally obtained from 10 ml of blood.

Key Words: Cellular immunity, Cytokine, Tumor associated antigen.

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DOES OVERNIGHT RESTING REALLY ENHANCE THE PERFORMANCE OF ELISPOT ASSAYS?

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Testing cryopreserved PBMC instead of fresh cell material is increasingly preferred in the field of immune monitoring. After the cells have been thawed, they can be plated in ELISPOT assays directly for testing. A modification of this protocol requires the cells to be kept in the incubator overnight before plating into ELISPOT assays for testing. There have been claims that "overnight resting" improves the signal to noise performance of the test result. "Resting", however, increases the duration of the assay, adds substantial additional labor, and causes loss of precious cell material. Driven by the hope of better assay performance, "resting" has become increasingly popular in the field of immune monitoring with ELISPOT, however, there have been no systematic studies published as yet to verify that resting indeed improves the assay performance. To this end, we are systematically testing cryopreserved PBMC from 70 donors in ELISPOT assays with and without "resting"; the result will be presented at the meeting.

Key Words: Cellular immunity, Cytokine, Tumor associated antigen.

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FIBROBLAST GROWTH FACTOR-1 INDUCES PRIMARY EPITHELIAL TUMOR CELL PLASTICITY TOWARD A MESENCHYMAL-LIKE PHENOTYPE

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Using a combination of culture conditions and tumor cell separation based on Neu and CD24 expression, we cloned both epithelial and mesenchymal-like tumor cells from spontaneous FVB MMTV/Neu mammary tumors. We determined that the epithelial cells were more tumorigenic when inoculated subcutaneously into FVB MMTV/Neu mice, whereas, the mesenchymal-like cells upregulated genes associated with inflammation and metastasis such as Zeb1, Zeb2, Snail, Slug, MMP9, TNF-α, FGF-1, CXCR4, IL-6 and Nos2. When a clonal population of epithelial tumor cells was cultured with fibroblast growth factor-1 (FGF-1), there was a significant increase in the expression of mesenchymal transcription factors Zeb2, Snail and Slug and an increase in mobility. Given these data, we hypothesize that primary tumor cells possess differentiation plasticity, and that the influence of external factors determine whether a cell is a tumor promoting epithelial or a metastatic mesenchymal-like cell. To test this hypothesis, we are comparing gene expression, proliferation, migration, and cytokine secretion in cloned primary epithelial and mesenchymal tumor cells exposed to FGF-1, and the growth factor receptor kinase inhibitor, TKI258. We anticipate that epithelial cells exposed to FGF-1 will upregulate mesenchymal transcription factors, will be less proliferative, more migratory and will secrete more immune-suppressive cytokines such as IL-1β, TNF-α, IL-6, and IL-10 than untreated cells. We also anticipate that addition of the TK1258 kinase inhibitor will reverse the transition to the mesenchymal-like phenotype induced by FGF-1. For in vivo studies, we have clonal luciferase expressing primary epithelial and mesenchymal tumor cell lines that we will test for metastatic potential. We anticipate that the mesenchymal tumor cells will be the most metastatic and that epithelial tumor cells will gain metastatic potential under the influence of FGF-1. We also predict that in vivo administration of TK1258 will reduce metastasis. Preventing the transition to a mesenchymal-like differentiation state may serve to reduce immune-suppressive factors secreted by tumor cells and may be required for optimal immunotherapeutic anti-tumor responses.

Key Words: Breast cancer, EGFR inhibitors, Tumor microenvironment.

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PRECLINICAL EVALUATION OF WITHAFERIN A AS AN ANTI-CANCER STEM CELL AGENT USING A UNIQUE HUMAN UP-LN1 CARCINOMA CELL LINE WITH CANCER STEM CELL PROPERTIES

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The operational definition of cancer stem cells (CSCs) describes CSCs as a subpopulation of cancer cells capable of efficiently seeding new tumors when inoculated into immunodeficient mice. Accumulating evidence has indicated that most anti-cancer therapies are effective against the bulk tumor cells, but not against CSCs. Previously, our group has identified withaferin A, a member of withanolides group of steroid compounds, able to inhibit both proliferation and reverse the epithelial-to-mesenchymal transition (EMT) process in cancer cells. In continuation, the aim of this study was to investigate whether withaferin A could be used to target CSCs and in turn reduce their proliferative activity, self-renewal potential and resistance against treatments. First, we isolated CD44high/CD24low floating cells (in the forms of cell aggregates/spheroids) originated from the UP-LN1 cell line in culture and subsequently verifed these floating cells to be the major niche of CSCs (Chen HC et al, Lab Invest 2011, June 20). The resulting floating cells could be serially subcultured and exhibited heightened stem-like properties including high self-renewal potential, formation of suspended cell aggregates and/or spheroids under serum-deprived conditions, resistance to multiple drugs and NL/LAK effectors, as well as an increased CDY1 (a novel iPS probe) retention. We found that withaferin A dramatically reduced the proliferation of the floating cell subset and their ability to form aggregates/spheroids in vitro. In addition, withaferin A markedly reduced the so-called side population (SP) cells (at 25μM) by 50%. Mechanistically, withaferin A treatment resulted in the down-regulation of STAT3-Mcl-1 axis which may play an important role in the development and maintenance of CSCs. While further refinement in the dose and schedule of withaferin A to maximize its efficacy and validation of the in vitro results by xenotransplantation studies are needed, we believe that this is the first study demonstrating the function of withaferin A to destroy the niche of CSCs.

Key Words: Chemotherapy.

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NATURALLY OCCURRING IMMUNE RESPONSES AGAINST SOX2 AND BCL-2 IN PATIENTS WITH ADVANCED AND EARLY-STAGE CANCER

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It has been documented by numerous reports that epitopes derived from human tumor-associated antigens can be recognized by the immune system of cancer patients. However, the majority of these antigens are not clinically relevant or important for cancer cell survival.

Moreover, in several animal and clinical immunotherapeutic studies it has been demonstrated that there is immune selection and immune escape against most of these antigens.

Deregulation of apoptosis proteins and pathways and death resistance of cancer "stem" cells have been observed in human cancer and often are due to over-expression of several proteins such as Bcl-2 and Sox2.

Sox2 and Bcl-2 are implicated in tumor progression, resistance and proliferation. The over-expression of these proteins in several tumors, acting as broad- spectrum antigens and the lack of relevant mutations makes them reliable targets for clinical immunotherapy trials.

Our research group designed several peptides representing potential immunogenic epitopes for both proteins. We identified spontaneous humoral and cellular immune responses against these Sox2 and Bcl-2-derived peptides in early-stage and advanced cancer patients suffering from ovarian, breast, pancreatic, colorectal, melanoma and sarcoma. In this study, we demonstrated that Sox2 and Bcl-2 could be targets for humoral and cellular immune recognition in cancer patients.

Furthermore, we describe naturally occurring immune responses against specific peptides derived from Sox2 and Bcl-2 in cancer patients by ELISA and ELISPOT, whereas no detectable responses were found in healthy patients. Thus, cellular and humoral immune responses against important proteins related to tumor survival like Bcl-2 and Sox2 seem to be common in cancer patients and could serve as broad-spectrum tumor antigens to achieve better clinical responses in cancer immunotherapy trials.

Key Words: Sox2 immune response, advanced cancer immune response, advanced cancer.

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AUTOLOGOUS VACCINE AHICE, THERAPY RESULTS

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AHICE immunotherapy is distinguished by its unique selectivity and specificity against recognized tumors. The peculiarity of AHICE is the demasking of the tumor cells biochemically. Following that the autologous immune system is being able to detect them spontaneously and eliminates them apoptotically.

We report here the AHICE therapy outcomes of different carcinomata.

AHICE was either sub cutan as a long term therapy of ninety days or i.v. administered as three up to ten infusions.

Before and after AHICE, every three weeks following were examined: A differential blood count, a lymphocytes immune-phenotyping, the related tumor markers, TNF-α-, IFN-γ-concentrations. At the end of AHICE the tumor situation was examined(MRI, CT or PET).

We found a confluence with good therapy outcome relatively (steady-state, or melting down of tumor or remission) at a level of minimum 1700 lymphocytes / µl in peripheral blood, a rising T4 in relation to T8 lymphocytes-concentration, an index T4/T8 of better than 1,5. The related tumor markers were at first rising up in respect of the strength of the immune response - this is for increasing cell-death apoptotically of the respective tumor cells and doesn't have to be interpreted as a progress of the tumor.

- A colon-ca. is still living without neoplasies at the best quality of life relatively, overcomes six years.

- A pancreas ca. after surgery treated first AHICE at 2000, lived without metas at the best quality of life until December 2009, that is rest life prolongation of nine years.

- A peritoneal ca. patient, with multifocal metas has after the first AHICE cycle a remission (CT) in June 2004 without neoplasies until December 2006.

- A breast cancer patient with Parkinson, treated first AHICE-cycle at 2005, is still living without neoplasies over six years at best quality of life.

- A small-cell lung-ca. with two brain metas and condition after a radiation treatment of that brain area and surgery of the lung tumors. Thereafter in 2009 start on the AHICE and during the over two years observation duration were no neoplasies noticed in the lung, liver as also the one brain-meta was melted down. The second brain-meta showed central a not agent incorporating area (CT, MRI, PET) but only in a small peripheral region of 1/4th. Because of the oedema an excision of the tumor was carried out. The immuno-histochemically examination showed multiple necrotic cells and increased CD56+ marker on cells (NKCs?). Therefore this is the proof of the in vivo effectiveness of AHICE.

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

Key Words: Autologous Vaccine AHICE, Immunotherapy, Cancer, Auto-Immune Diseases.

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PARTIAL CD4-DEPLETION ENHANCES THE EFFICACY OF MULTIPLE VACCINATIONS

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Few immunotherapists would accept the concept of a single vaccination inducing a therapeutic anti-cancer immune response in a patient with cancer. But what is the evidence to support the "more-is-better" approach of multiple vaccinations? Our group reported that T cells from mice thrice vaccinated with a GM-CSF secreting B16 tumor vaccine (D5-G6) were significantly less effective in adoptive transfer studies than T cells from mice receiving a single vaccination[1]. A striking difference observed in multiply vaccinated animals was an increase in the number of Tregs, that when partially depleted with anti-CD4 mAb restored therapeutic efficacy. However, we questioned whether tissue-resident tumor-specific T cells might have been missed in our adoptive transfer studies. To address this issue we used a protective vaccine model to test if thrice-vaccinated mice would reject a large tumor challenge ($20\times TD_{100}$). This was not the case with 100% of mice (8 of 8) immunized 3-times growing tumor. Again, Tregs increased with more vaccinations. Partial depletion of CD4 T cells 1-day prior to the 2nd and 3rd vaccination increased survival to 33% (3 of 9) ($p<0.006$). This CD4-depletion correlated with an increased ratio of CD8 to CD4FOXP3+ cells with each subsequent vaccination, compared to non-depleted mice. Fourteen days after the second vaccination CD4-depleted mice had a larger proportion of proliferating (Ki67+) FOXP3-negative CD4 T cells and lower frequency of Ki67+ "induced" HELIOS-negative Tregs compared to non-depleted mice. Suggesting a skewing of the T cell repertoire from immunosuppressive to activated. We also examined whether location of immunization altered vaccine efficacy. We compared two strategies: in one the total vaccine dose (5×10^6 D5-G6) was administered at 1-site, which rotated to a different limb for each vaccination. The second split the dose into 4 aliquots (1.25×10^6), administered to each limb for each vaccine. Fourteen days after the 3rd vaccination mice were challenged. The frequencies of B cells, macrophages and DCs were increased in the dLN and spleen 14 days after the initial immunization in mice vaccinated at 4-sites versus 1-site. However, there were no significant differences between protection [41% (5 of 12) versus 36% (4 of 11) survival] or the frequency of Tregs or MDSCs. Overall this data suggests that partial depletion of CD4 T cells early during immunization improves vaccine efficacy and provides support for the use of partial CD4-depletion as a potential strategy for combination therapy of patients with cancer.

1. LaCelle M, et al. Clin Can Res. 2009;15:6881-6890.

Key Words: multiple vaccinations, immunization, regulatory T cells.

Presenting author underlined. *Primary author in italics.*

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T-CELL IMMUNITY IN HEPATOCELLULAR CARCINOMA AGAINST THE WILMS' TUMOR PROTEIN-1 (WT1) AS A POTENTIAL TARGET STRUCTURE FOR IMMUNOTHERAPEUTIC APPROACHES

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Introduction: Immunotherapy approaches are currently proposed in hepatocellular carcinoma (HCC) and appear to be promising in treatment and prevention of HCC. One attractive target antigen for immunotherapy in HCC is the transcription factor Wilms tumor gene product 1 (WT1), which is overexpressed in HCC and may play an important role in carcinogenesis and prognosis of HCC. We analyzed WT1 specific T-cells spontaneously occurring in patients with HCC and patients with chronic hepatitis B and C (CH), or liver cirrhosis (LC). Methods: Using flow cytometry we analyzed WT1 specific CD4+ and CD8+ T-cells, regulatory T-cells (Treg) and myeloid-derived suppressor cells (MDSC) in peripheral blood (PB) after stimulation with WT1-peptide mix in 21 HCC patients, 21 CH patients, 23 LC patients, and in 9 healthy controls.

Results: WT1-specific IFNy+CD8+ T-cells were significantly elevated in patients with HCC (mean 0,74%), CH (mean 0,37%), and LC (mean 0,22%) in comparison to healthy controls (mean 0,15%), whereas no significant difference was observed between the disease groups. The frequency of WT1-specific IFNy+CD4+ T-cells in HCC patients (mean 0,74%) was significantly higher than in CH patients (mean 0,21%) and healthy controls (mean 0,11%). For both WT1-specific CD4+ and CD8+ T-cells we demonstrated a higher expression of the activation marker CD137 in HCC and LC than in CH patients. Regarding the frequencies of CD25+CD127- Treg a significant difference was seen in patients with HCC, CH, and LC compared to healthy controls. Furthermore, we were able to demonstrate significantly elevated frequencies of MDSCs in HCC patients compared to those in patients with CH and LC, and healthy controls.

Conclusion: These results provide initial evidence for spontaneous T-cell reactivity against WT1 in HCC patients and in patients with chronic liver disease. This data supports the immunogenicity of WT1 in HCC and the potential usefulness of this antigen for HCC immunotherapy. However, the detection of enriched frequencies of Treg and MDSC suggests the need for special attention and better understanding of immunosuppressive mechanisms in HCC.

Key Words: CD8+ T cells, Myeloid derived suppressor cell, Treg cells.

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THE POST-PROLINE CLEAVING ENZYME INHIBITOR 4175 IS A NOVEL IMMUNOTHERAPEUTIC ADJUVANT

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We have previously shown that PT-100, a pan-inhibitor of a subset of post-proline cleaving enzymes (PPCEs) known as dipeptidyl peptidases (DPP), induces complete regression of solid tumors in a T cell dependent manner due to accelerated tumor-specific T cell priming and increased dendritic cell trafficking to the tumor draining lymph node. 4175 is a novel second generation PPCE inhibitor capable of rejecting bulky solid tumors in mice with improved therapeutic index. In a dose escalation study in female C57BL/6 mice, 4175 was well-tolerated and anti-tumor activity was observed between 50ug and 200ug doses. RMS M3-9-M challenged mice that regressed after 4175 treatment were rechallenged with 5x105 RMS M3-9-M cells and given no additional treatment. These survivors effectively rejected tumor compared to naïve mice (n=7, $p<0.001$) without additional 4175 treatment, indicative of immunological memory. When combined with DC vaccine pulsed with irradiated tumor cells, late treatment with 4175 induced complete regression of well-established RMS M3-9-M (10 days post injection) in 70% of mice compared to 30% in non-vaccinated 4175 treated mice (n=7, $p=0.0379$). When combined with adoptive transfer of T cells from vaccine-primed donors, 4175 did not significantly improve survival as compared to 4175 alone, however tumor size was reduced. In an attempt to identify targets of 4175 activity, selective inhibitors targeting specific DPPs (DPPII, DPPIV, DPP8, DPP9, FAP) were tested and found to be ineffective as single agents. When inhibitors were combined in a cocktail and administered to tumor-bearing mice, cocktail treated mice had significantly larger tumors compared to 4175 treated mice (n=7, $p<0.001$), suggesting other PPCE targets are likely responsible for the anti-tumor activity of 4175. In summary, PPCE inhibition using 4175 results in tumor regression, immunologic memory and immunotherapeutic adjuvant effects. Single-target inhibition studies suggest that DPPs may not be the primary targets mediating anti-tumor effects.

Key Words: Cell trafficking, Combination immunotherapy.

Presenting author underlined. *Primary author in italics.*

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IFN-ALPHA INCREASES THE CYTOTOXIC EFFECT OF CIK CELLS ON B-ALL

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Haematopoietic stem cell transplantation (HSCT) is required in about 20-30% of children with B-lineage acute lymphoblastic leukemia (B-ALL). Relapses after HSCT are usually refractory to further therapy and in these cases, the development of an optimized immunotherapeutic strategy would be of great clinical interest. In this setting, the Cytokine-Induced Killer (CIK) cells could represent an interesting tool for immunotherapy. Indeed, they were showed to be highly cytotoxic against many cancer types. Nevertheless, their cytotoxicity against ALL cells is not consistent. Therefore, we have investigated the possibility of combining adoptive immunotherapy with CIK cells and interferon alpha (IFNα), to optimise the cytotoxicity of CIK cells against B-ALL cells.

CIK cells were differentiated from cord blood mononuclear cells or peripheral blood mononuclear cells for 21 days. At the end of the culture, there were around 45% CIK cells (CD3+CD56+). The other cells were 1% natural killer (NK) cells and 54% T cells. The bulk CIK (CIK cells, NK cells and T cells) showed a mild cytotoxic activity against B-ALL cell lines. However, when the bulk CIK was purified with CD56 human microbeads there was significant cytotoxic activity against B-ALL cell lines. In addition, we have showed that sorted CIK cells removed from NK and T cells, always showed a cytotoxic activity against B-ALL cells lines. Also, after pre-incubation of sorted CIK cells with IFNα overnight, we have observed an increase of cytotoxicity by more than 20-40%. CIK cells displayed a phosphorylation of STAT-1 after stimulation by IFNα.

In addition, we have tested in vivo CIK cells in NOD/SCID/γc- (NSG) mice injected with human B-ALL cell lines and we could show that CIK cells (Target on effector ratio of 1:80) could significantly delay mice mortality. Also, we showed that CIK cells treated by IFNα did not the induce of xeno-Graft-versus-Host Disease (GvHD) in NSG mice.

In conclusion, we showed that CIK cells are cytotoxic against B-ALL when they are purified and also their effect is increased by the IFNalpha via STAT-1. Finally, the CIK cells have a GvL effect (graft versus leukemia) in the NOD/SCID/γc- mouse model.

Key Words: CIK cells, IFNalpha, B-ALL.

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INNATE IMMUNE ACTIVATION AND VACCINE ADJUVANT POTENTIAL OF TLR-2 AGONIST, POLY SACCHARIDE KRESTIN (PSK)

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Toll-like receptors have been pursued as targets for vaccine development due to their stimulation of an antigen specific response in addition to an innate immune response. While TLR-7, -8, and -9 have been explored most thoroughly, other TLR agonists are being developed for many different diseases including cancer therapies. Polysaccharide kresin (PSK) is obtained by hot water extraction of the Trametes versicolor mushroom and has been extensively studied in Asia, especially for gastrointestinal cancer. Our previous studies have shown that PSK selectively activates TLR2 and can decrease tumor volume in implanted and spontaneous tumor in the neu-transgenic murine breast cancer model. The current study was undertaken to investigate the effect of PSK on innate immune cells, specifically natural killer (NK) and dendritic cells (DC), as well as T-cells for the potential of using PSK as a vaccine adjuvant. We found PSK can activate NK cells demonstrated by increased expression of CD69 and CD25, and enhanced production of IFN-γ. PSK activates DCs in vitro and in vivo shown by increased expression of CD80, CD86, MHC-II, and IL-12. When PSK was administered as an adjuvant to Ova p323 peptide vaccine, it stimulates the proliferation of adoptively transferred ova-specific CD4+ T-cells and results in increased number of antigen-specific CD4+ T-cells. Together, these data suggest the potential of using PSK, a natural product with TLR2 agonist potential, to activate DC and enhance both innate and antigen-specific adaptive immune response. Further investigation of the role of PSK as an adjuvant in combination immunotherapy with HER2-targeted monoclonal antibody and peptide vaccine is on-going.

Key Words: Cancer vaccine, Combination immunotherapy, Innate immunity.

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RADIATION AND ENDOPLASMIC RETICULUM STRESS-INDUCER PROMOTE CALRETICULIN TRANSLOCATION, CONTRIBUTING TO IMMUNOGENIC CELL DEATH OF CANCER CELLS

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We hypothesize that cell damage and death from the effect of ionizing radiation (IR) and endoplasmic reticulum (ER) stress-inducing agents could: 1) be monitored in vitro; and 2) contribute to an anti-

tumor immune response via the induction of mediators of immunogenic cell death (ICD) of cancer cells. ICD promotes the cross-presentation of tumor-derived antigens by dendritic cells (DCs) to T cells (Semin Immunol 22, 113-124, 2010). Calreticulin (CRT, an ER chaperone protein) redistribution to the surface of tumor cells acts as a potent "eat me" signal for DCs involved in tumor associated antigen processing, thereby serving as a key step in ICD. In the clinical setting, IR or ER stress alone may not quantitatively and/or qualitatively achieve tumor cell death in a manner that specifically triggers immune-mediated tumor rejection. Thus, we hypothesized that clinically relevant doses of IR, when combined with thapsigargin (Tg, an ER stress-inducer via sarcoplasmic/ER calcium ATPase inhibition), may intensify CRT translocation to the cell surface. To test this, we employed the poorly immunogenic 4T1 mouse breast cancer cells. 4T1 cells were treated with IR (0, 6, or 20 Gy) followed by 24 hrs. culture in the presence or absence of Tg (1 mM). Thereafter, the cells were assayed either via Western blot (WB) or immunofluorescence (IF). Cytotoxicity was determined via MTT assay at 12, 24, and 48 hrs. Relative amounts of protein were determined via WB analysis with specific antibodies to phospho-EIF2-a, caspase-8, BAP-31, and PARP. Actin was used as a loading control. CRT redistribution was determined by IF analysis. When combined, IR (6 Gy) + Tg (1 mM) triggered elevated phosphorylation of EIF2-a (a marker for ER stress and protein translation inhibition) in 4T1 cells. In addition, IR (6 and 20 Gy) + Tg (1 μM) increased the cleavage of the apoptotic markers caspase-8, BAP-31, and PARP. Finally, we observed that cell death by IR (6 and 10 Gy, single dose) in the presence of Tg (1 μM) was preceded by enhanced CRT translocation to the cell surface. In this in vitro model, IR (6Gy and 10 Gy) alone was unable to incite CRT redistribution. However, in the presence of Tg (1 μM), IR (6Gy) CRT redistribution occurred and was superior to controls. Taken together, these findings suggest that IR combined with an ER stress-inducing agent is a novel application of radiotherapy that can potently trigger ICD and serve as a strategy to promote immune-mediated tumor rejection in cancer patients.

Key Words: radiotherapy, ER-stressors, calreticulin.

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HSP90 INHIBITORS ENHANCE IMMUNOLOGICAL TARGETING OF MELANOMAS

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As part of an ongoing effort to identify compounds that enhance tumor antigen recognition, we utilized a cell-based screening assay to demonstrate increased T cell recognition of melanoma cells that were treated with a series of more than 600 known biologically active compounds. The goal of this study is to provide a combination therapy where immunological intervention, such as vaccines or adoptive immunotherapies, will have improved efficacy if the tumors are also induced to express the targeted antigens. From the screen, 9 "hit" compounds showed desirable tumor antigen-enhancing properties with retained T cell function in the presence of the treatment compound. Among the most active of the "hits" were the HSP90 inhibitor, 17-AAG, and the anti-metabolite, aphidicolin. In follow-up, a total of 12 different HSP90 inhibitors (iHSP90) were tested, including many that are already in human clinical trials as single agents, and each was active in inducing a variety of melanocytic differentiation antigens that allow improved T cell recognition of the treated tumor cells. We observed enhanced Melan-A/MART-1 expression, as well as increased gp100 and TRP-2 expression on a variety of melanoma cell lines that included cells that are known to have activating mutations in BRAF, as well as BRAF wild-type tumor cells that contain NRAS mutations. Our investigations indicate that protein levels of the melanocyte differentiation antigens are increased cytoplasmically, as indicated by increased staining with antibodies to these proteins, as well as enhanced activity of the Melan-A/MART-1 promoter. Our studies indicate that B-raf protein expression is diminished by the iHSP90. Together with the promoter studies, these data suggest that the client proteins for HSP90 may be signal transduction or gene regulatory proteins rather than the targeted antigens themselves. In addition, we noted that surface MHC Class I antigen expression is enhanced in a variety of tumors, including melanomas, gliomas, breast cancer, osteosarcoma, cervical cancer and lymphomas. These studies indicate that iHSP90 treatment improves tumor antigen recognition on a variety of tumors, both through enhanced expression of differentiation antigens, and through induction of MHC antigens, allowing for T cells to recognize tumor cells that might otherwise escape immune detection. The use of such drugs in combination immunotherapy of human cancers is an exciting prospect for enhancing therapeutic outcomes.

Key Words: Combination immunotherapy, Melanoma, Tumor associated antigen.

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RNACTIVE VACCINES PROVIDE STRONG ANTI-TUMOR EFFECT ESPECIALLY IN COMBINATION WITH ANTI-CTLA4 THERAPY OR RADIATION

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Based on modified and protamine-formulated messenger RNA, two-component RNActive vaccines combine two principle activities: high antigen expression and immune stimulation mediated by TLR7. That they do not need any additional adjuvant to be active and can be administered via single intradermal injection, supports their unique potency. RNActive vaccines induce innate as well as balanced and long-lasting adaptive immune response and provide strong anti-tumor effect.

To increase the therapeutic effect, potentially allowing for the control of large established tumors, we combined RNActive vaccines with two different standard therapies.

E.G7-OVA bearing mice were treated therapeutically with either OVA- RNActive alone or in combination with anti-CTLA4 antibody. Combination therapy resulted in a strong, synergistic anti-tumor effect, yielding a higher frequency of mice with complete tumor rejection. Remarkably, the complete responders were protected against rechallenge with parental ovalbumin-negative EL-4 tumors, indicating an antigen spreading in this group. Interestingly, following combination therapy, tumors which escaped the control of the immune system exhibited strongly reduced ovalbumin expression.

Next we tested whether combination of RNActive vaccines with radiotherapy can inhibit the growth of established, low immunogenic and radiation-insensitive, Lewis lung carcinoma (LLC) tumors. Immunotherapy alone, targeting two tumor/self-antigens, was not effective in inducing tumor protection. High dose radiation of the tumors induced only transient growth stagnation. However, combined radioimmunotherapy dramatically improved anti-tumor efficacy and supported surveillance of large tumors in treated mice. To understand the cellular and mechanistic base for the synergistic effect, we conducted a comprehensive flow cytometric analysis of LLC tumor tissues one week after treatment began. Although at this time point the tumor volume was still comparable in all mice, we observed dramatic changes in the cellular composition of the tumor tissue. Tumors from mice receiving radiation and combination therapy were highly infiltrated by immune cells; the frequency of cells associated with adaptive immunity was particularly increased in mice treated with combination therapy.

These findings highlight that combination of RNActive vaccines with standard cancer therapies, such as antibody therapy or radiation, creates highly synergistic anti-tumor effects, which may have the potential to improve long-term survival in cancer patients.

Key Words: Animal model, Cancer vaccine, Combination immunotherapy.

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IDO PEPTIDE VACCINATION TRIAL FOR PATIENTS WITH METASTATIC NON SMALL-CELL LUNG CANCER. A FIRST-IN-MAN PHASE I TRIAL (NCT01219348)

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Background: Indoleamine 2.3-dioxygenase (IDO) is an immune regulatory enzyme that suppresses T-cell immunity. We have previously demonstrated that cancer patients elicit a spontaneous IDO-peptide specific T-cell response with anti-tumour activity. Based on these results we have initiated a clinical investigation of an IDO peptide vaccine in patients with non small-cell lung cancer (NSCLC). Since IDO expression is seen in both cancer- and antigen presenting cells the vaccination against IDO expressing cells is two-sided.

Methods: We included 13 patients with incurable stage III-IV NSCLC off chemotherapy. Patients were treated with IDO peptide vaccine and the adjuvant Montanide plus Imiquimod ointment. The IDO peptide (ALLEIASCL) is HLA-A2 restricted thus only A2 positive patients were eligible. The vaccine was given subcutaneously, and patients were evaluated by CT-scan (RECIST 1.1) every 3rd month. Patients were treated until progression. Primary end point was toxicity, secondarily clinical and immunological responses were assessed.

Results: Patient characteristics: Mean age =63 (range 49-70), sex (F=6, M=7), PS (0=7, 1=6), histology (sqamous/mixed subtype=2, non-squamous=11). Number of previously received chemotherapy regimens: (1st line of chemotherapy =100%, 2nd line=38% and 3rd or more regimens =15% of the patients). Safety evaluation demonstrated no grade 3-4 CTCAE (v.3.0) toxicity. To date 4/9 patients have responded on an indirect IFN-y Elispot (assays done in duplets of 2 concentrations; a minimum of a 2 fold increase was regarded as a response). At the 1st CT scan evaluation stable disease (SD) was seen in 60% of the patients. In average the patients with SD have received 12 vaccines and have obtained duration of SD for 8.3 months.

Conclusion: The vaccine is safe and well-tolerated. Immune responses in Elispot assays are seen in 44% of the patients. SD is seen in 60% of patients at 1st evaluation and to date the average duration of SD is 8.3 months.

Key Words: Cancer vaccine, Lung cancer, Phase I.

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DIFFERENTIAL RESPONSES TO IMMUNOTHERAPY OF ORTHOTOPIC TUMORS COMPARED TO SUBCUTANEOUS TUMORS

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Established subcutaneous renal cell carcinoma tumors can be eradicated in mice using immunotherapies consisting of various combinations of antibodies specific for CD40, CD137, DR5 and the TLR agonist CpG. A major role for CD8 T cells with a contribution from NK cells has been determined for this effect. However, we have observed that these same immunotherapies were much less effective against orthotopic disease implanted in the kidney. Approximately 80% of subcutaneous tumors were eradicated whereas less than 10% of kidney tumors fully regressed. To investigate the reasons for these differential responses, we examined various tumor-intrinsic and tumor-extrinsic parameters. Tumors in both locations were of similar size and expressed similar levels of DR5, CD40 and CD137. In addition, blood vessel density and permeability was similar in renal and subcutaneous tumors. When cellular composition of tumors was analyzed, a higher frequency of F4/80hiCD11blo macrophages was detected in orthotopic tumors. The composition of tumor-draining lymph nodes was similar in mice bearing subcutaneous or renal tumors, with the exception of dendritic cell subsets that were elevated in inguinal nodes draining subcutaneous tumors

following treatment. Preliminary experiments suggest that differences in the cytokine microenvironment exist between subcutaneous and renal tumors, with the macrophage-attracting chemokine MCP-1 expressed more highly in kidney tumors, and IFN-γ expressed more highly in subcutaneous tumors. MHC class I was also more highly expressed on subcutaneous tumor cells. These data imply that differences in the local tumor microenvironment lead to a relatively greater anti-tumor immune response in subcutaneous tumors. These observations demonstrate that tumors in different anatomical locations can differ markedly in their responses to immunotherapy, and suggest that immunotherapies should be tested in appropriate mouse models of malignancy.

Key Words: Combination immunotherapy, Macrophages, Tumor microenvironment.

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DOCETAXEL ENHANCES THE EFFICACY OF DC IMMUNOTHERAPY BY REDUCING MYELOID DERIVED SUPPRESSOR CELLS IN MELANOMA MODEL

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Melanoma is considered to be an epidemic, with one American dying from melanoma every 60 minutes. Factors such as T regulatory cells and myeloid derived suppressor cells (MDSC) limit the effectiveness of current immunotherapy protocols. Hence, the multiple immunotherapeutic approach is required for the induction of effective anti-tumor immunity. Several reports have shown that administration of chemotherapeutic agents or total body irradiation (TBI) before adoptive transfer of tumor-specific T cells reduces or eliminates the immunosuppressive populations such as T regulatory cells and myeloid derived suppressor cells. However, in our preliminary data we observed that the MDSC reconstitution during homeostatic proliferation was very rapid. By day 10, MDSCs were fully reconstituted in the TBI treated B16 tumor bearing mice and were also found to be highly immunosuppressive compared to the control mice with no TBI treatment. In an effort to block their rapid reconstitution and their immunosuppressive function in the TBI treated tumor bearing mice, we treated the mice with four doses of docetaxel (16.5mg/ kg/ body weight), a known chemotherapeutic agent that selectively targets MDSCs. By day 10, 100% reconstitution of MDSCs in the spleen was observed in the control mice while approximately 60% reduction in the MDSC reconstitution was detected in the spleen of mice that received docetaxel treatment every 3-4 days. We also measured reconstitution of the immune subsets such as CD4, CD8, or T regulatory cells. We observed a slight decrease in the CD4 and CD8 population in the docetaxel treated mice. However no statistical significance was observed between the groups. Based on these results, we evaluated the efficacy of DC immunotherapy in combination with or without docetaxel in the TBI treated mice bearing B16 melanoma. After 4 weeks of treatment, DC immunotherapy in combination with docetaxel significantly inhibited the tumor growth (62mm2) compared to the DC (230mm2) or DTX (147mm2) alone treated mice. Also, considerable reduction in the MDSC population (12.34%) was observed in the spleen of mice that received a combination treatment and also in the DTX treated mice (17.21%) compared to the control group (35.2%). Docetaxel treatment enhanced the survival rate in the DC vaccinated mice compared to DC or docetaxel alone treated mice. We also observed the selectively increased CTL response in the splenic T cells of mice that received a combined treatment (32% lysis) compared to the DC (13% lysis) or DTX alone (15% lysis) or a control group (7% lysis) against B16 melanoma. These findings suggest that docetaxel might have a clinical benefit when combined with immunotherapy for the treatment of melanoma.

Key Words: Combination immunotherapy, Dendritic cell, Melanoma.

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ENHANCED EXPRESSION OF CANCER-TESTIS ANTIGENS IN PROSTATE CANCER CELL LINES TREATED WITH EPIGENETIC MODIFYING AGENTS

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The Cancer-Testis Antigen (CTA) family of genes are characterized by tissue-restricted expression to MHC class I deficient germline cells and ectopic expression in numerous types of malignancies. The restricted expression pattern of CTAs suggests that they may be ideal immunotherapeutic targets for vaccine based therapies. However, the pattern of expression of CTAs in malignant tissues is quite heterogeneous and regulated, in part, by epigenetic events. The goal of this investigation was to identify potential target antigens for immunotherapeutic approaches as well as the inducibility of these antigens. This study was designed to characterize the expression of 31 CTAs in 6 prostate cancer (PRCA) cell lines (DU145, LNCaP, 22RV1, LAPC4, PC3, VCaP) and 1 prostate epithelial line (RWPE-1) and the ability of epigenetic modifying agents, 5-aza-2'-deoxycytidine (5AZA) alone or in combination with the histone deacetylase inhibitor, LBH589 (Panobinostat), to modulate expression of these genes. All cell lines were treated with vehicle or 5AZA at 10μM for 72 hours and LBH589 at 10 or 100nM was added during the final 24 hours. Total RNA was then harvested from these cells, reverse transcribed to cDNA and subjected to quantitative PCR for analysis of gene expression. Expression patterns of 12 CTAs in 5 cell lines have been completed to date. This analysis has identified subsets of CTAs that are not expressed in PRCA cell lines (ADAM2, SSX3, SSX5), expressed at baseline in multiple PRCA cell lines (SSX2), and variable baseline expression between cell lines (NY ESO, MAGE A1, MAGE A3). Interestingly, different subsets of genes were identified that are inducible following treatment with 5AZA but not LBH589 (MAGE A3, MAGE B1), induced by LBH589 alone (MAD CT-2), and induced by the combination of 5AZA and LBH589 (NY ESO, NYSAR-35). In the subset of genes not expressed at baseline, ADAM2 was not inducible in any cell line studied to date while SSX 3 and SSX5 were

not expressed at baseline but induced following treatment with 5AZA. This work indicates that numerous family members in the CTA family are expressed in prostate cancer cell lines and supports the development of certain subsets of the CTA family as target antigens for immunotherapies in prostate cancer. Furthermore, treatment with epigenetic modifying agents may increase antigen expression and presentation of unique subsets of CTAs in prostate cancer cells, suggesting a potential role for this class of drugs in combination with immunotherapies.

Key Words: Prostate cancer, Targeted therapeutics, Tumor associated antigen.

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HISTONE DEACETYLASE INHIBITOR OSU-HDAC42 ENHANCES THE ANTIGEN-SPECIFIC T CELL IMMUNE RESPONSES AND THERAPEUTIC ANTITUMOR EFFECTS GENERATED BY THERAPEUTIC HPV DNA VACCINE

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Multimodality treatments that combine conventional cancer therapies with antigen-specific immunotherapy have emerged as promising approaches for the control of cancer. Antigen-specific immunotherapy is an attractive approach for the treatment of cancers since it has the potency to specifically eradicate systemic tumors and control metastases without damaging normal cells. A favorable approach for antigen-specific immunotherapy is the use of DNA vaccines due to their safety, stability and ease of preparation. We have previously developed therapeutic human papillomavirus (HPV) DNA vaccines encoding calreticulin (CRT) linked to HPV-16 E7 antigen (CRT/E7) to enhance therapeutic HPV DNA vaccine potency. The CRT/E7 DNA vaccine has been tested in early phase clinical trials. In the current study, we explore the employment of a new histone deacetylase (HDAC) inhibitor, OSU-HDAC42, in combination with CRT/E7 DNA vaccine in a preclinical model for its potential to enhance E7-specific CD8+ T cell immune responses as well as antitumor effects against E7-expressing tumors. OSU-HDAC42 has also been in use in early phase clinical trials. We found that treatment with OSU-HDAC42 led to upregulation of MHC class I molecules in E7-expressing TC-1 tumor cells, rendering the tumor cells more susceptible to E7-specific CD8+ T cell-mediated killing. In addition, treatment with OSU-HDAC42 enhanced the expression of the protein encoded by the DNA construct in transfected dendritic cell line DC-7. More importantly, treatment with combination of OSU-HDAC42 and CRT/E7 DNA vaccine led to improved antigen-specific CD8+ T cell immune responses as well as therapeutic antitumor effects in TC-1 tumor-bearing mice. Thus, our data indicate that OSU-HDAC42 can enhance therapeutic HPV DNA vaccine potency in generating improved antigen-specific immune responses and antitumor effects. These findings have important implications for future clinical translation.

Key Words: Cancer vaccine, Combination immunotherapy, HPV.

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VTX 2337, A SELECTIVE TLR8 LIGAND, ENHANCES CETUXIMAB-MEDIATED ADCC IN HEAD AND NECK CANCER

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Aim: To determine the potential enhancement in cetuximab-mediated antibody dependent cytotoxicity (ADCC) of HNC cells by toll-like receptor 8 (TLR8)-stimulated human PBMC and to correlate these results with FcγR IIa polymorphisms on monocytes (codon 131 H/R) and FcγR IIIa polymorphisms on NK cells (codon 158 V/F).

Methods: Cytotoxic activity of PBMC or isolated NK was determined using a Cr-51 release assay during which PBMC pre-treated with TLR8 agonist VTX 2337 (250 nM 18 h) were incubated with EGFR+ UM-22B HNC cells and cetuximab (10 μg/mL) for 4 h. PBMC cytokine release was also measured using a multiplex cytokine assay. FcγR IIIa-158 genotype was determined using a quantitative PCR-based assay kit. Significant differences in ADCC by FcγR IIIa or TLR8 genotype were determined using a Kruskal-Wallis one way analysis of variance, and a post hoc Mann-Whitney non-parametric t test was performed for differences between groups. For cytokine analysis, a Mann-Whitney non-parametric test was performed.

Results: TLR8 stimulation enhanced ADCC lytic activity in NK expressing FcγR IIIa FF genotype (2.6-fold), VF (1.7-fold) and VV (1.8-fold), with significant difference between genotypes ($p=0.0389$). Enhancement was significantly greater in FcγR IIIa FF genotype than NK with a V allele ($p=0.0130$). NK cells were the primary effectors in VTX 2337-enhanced, cetuximab-mediated ADCC. Th1 cytokines were secreted at significantly higher levels in VTX 2337-treated PBMC versus untreated PBMC: TNF-α ($p<0.0001$), IFN-γ ($p<0.0001$), IL-12p40 ($p=0.0002$), and IL-12p70 ($p=0.03$). TLR8 SNP (TLR8 A1G) did not influence the lytic activity seen on cetuximab-mediated ADCC ($p=0.7153$). TLR8 stimulated PBMC from HNC patients were more avid effectors in cetuximab-mediated ADCC ($p=0.0262$), and this treatment caused an additional subset of HNC patient PBMC to manifest cetuximab-mediated ADCC.

Conclusion: VTX 2337 enhances cetuximab mediated ADCC against HNC cells, and can induce cetuximab-mediated lytic activity

in PBMC from HNC patients that did not initially mediate ADCC. Three key Th1 cytokines (TNF- α, IFN- γ and IL-12) are preferentially stimulated by VTX 2337 treated PBMC.

Key Words: ADCC, Adjuvant, Combination immunotherapy.

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IMMUNOMODULATORY EFFECTS OF DASATINIB SYNERGIZE WITH CTLA-4 BLOCKADE RESULTING IN ENHANCED ANTITUMOR ACTIVITY

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Dasatinib is a highly potent BCR-ABL inhibitor used for the treatment of patients with chronic myeloid leukemia (CML). Exposure of immune cells to Dasatinib in vitro has been associated with immunomodulatory effects on T-cells and CD11b+ myeloid cells. We hypothesize that activity of Dasatinib in vivo is in part related to its immunomodulatory effects. The purpose of these studies was to determine the antitumor activity and immunomodulatory effects of Dasatinib as monotherapy and in combination with CTLA-4 blockade in vivo. Efficacy studies were conducted in five solid tumor models: P815 mastocytoma, SA1N fibrosarcoma, CT-26 colon carcinoma, M109 lung carcinoma, and B16/F10 melanoma. Dasatinib was administered at 30 mg/kg BID (twice a day) on a 5-day on/2-day off schedule for a total of three dosing cycles or daily for 15 consecutive days; CTLA-4 mAb was dosed at 20 mg/kg every fourth day for three doses. CTLA-4 mAb was active in the P815, SA1N, and CT-26 models, with minimal activity against the M109 model and no activity observed against B16/F10. Dasatinib dosed intermittently (5 day on/2 day off) showed minimal activity against P815 and SA1N models and was not active against CT-26, M109 or B16/F10. Concurrent treatment with Dasatinib + CTLA-4 mAb resulted in additive or synergistic effects in the SA1N, P815 and CT-26 tumor models. In the M109 and B16/F10 models where both Dasatinib and CTLA-4 blockade are inactive, no antitumor effect was observed. Additional studies were conducted in the CT-26 tumor model to determine whether the enhanced antitumor activity was due to an expansion of cytotoxic T cells and whether the treatments were altering the composition of the immune cells in the tumor-draining lymph nodes and tumors. Increased in vivo cytotoxic activity against a CT-26 peptide was observed in animals treated with the combination treatment compared to animals treated with single treatments ($p<0.05$), which correlated with an increase in the ratio of activated CD8+ effector cells over CD4+FoxP3+ T cells. Dasatinib therapy also induced changes in the composition of T cells promoting a more favorable ratio of T effectors/T regulatory cells compared to controls. In addition, microarray gene analyses of tumors treated with the combinatorial approach demonstrated marked changes on pathways involved with immune function. Thus, Dasatinib modulates the composition of immune cells in the tumor-draining lymph nodes and tumor microenvironment to promote enhancement of antitumor immune responses in combination with CTLA-4 blockade.

Key Words: CTLA-4, Dasatinib, Immunomodulation.

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USE OF COMPLEMENTARY AND ALTERNATIVE MEDICINE IN PATIENTS WITH HAV/HBV/HCV INFECTIONS: RESULTS FROM A CROSS-SECTIONAL STUDY IN THE SHERPUR DISTRICT OF BANGLADESH

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Hepatitis A, Hepatitis B, and Hepatitis C are viruses (HAV/HBV/HCV), which causes HAV/HBV/HCV infections. HAV/HBV/HCV is one of the world's most common infectious diseases. Infections can lead to severe liver diseases, which may last throughout a patient's life. Around 25% of carriers will develop serious liver diseases, including chronic hepatitis, liver cirrhosis, and primary liver cancer. More than one million deaths per year are recorded due to HAV/HBV/HCV infections. HAV/HBV/HCV is the most common diseases in Bangladesh. The objective of this study was to conduct a survey amongst the local specialists in the Sherpur district of Bangladesh, to collect information on plants used to treat HAV/HBV/HCV infections. Local specialists of the study area were selected randomly and interviewed with the help of translators to gather information on the knowledge and use of plants used as a remedy for HAV/HBV/HCV infections. In-depth information regarding plants type, preparation of medicines, ailments for which they are used, dosages, and side effects if any, were obtained from the local specialists. All plants were photographed, collected, identified, and vouchers were stored at the Bangladesh National Herbarium. Information on thirty-six plants was obtained. The collected information indicates that the following plants are used to treat HAV/HBV/HCV infections: Lawsonia inermis L., Terminalia arjuna (Roxb. ex DC.) Wight & Arn., Terminalia bellirica (Gaertn.) Roxb., Sesamum indicum L., Terminalia chebula Retz., Cicer arietinum L., Swertia chirata Buch.-Ham. ex Wall., Abrus precatorius L., Daucus carota L., Citrus aurantiifolia (Christm.) Swingle, Aloe vera (L.) Burm.f., Phoenix sylvestris (L.) Roxb., Dillenia indica L., Ocimum gratissimum L., Cocos nucifera L., Saccharum officinarum L., Piper nigrum L., Andrographis paniculata (Burm.f.) Nees, Aegle marmelos (L.) Corrêa, Diospyros malabarica (Desr.) Kostel., Vitis vinifera L., Curcuma amada Roxb., Limonia acidissima L., Carica papaya L., Scoparia dulcis L., Azadirachta indica A.Juss., Boerhavia diffusa L., Lepidagathis hyalina Nees, Nigella sativa L., Aconitum napellus L., Agaricus campestris L., Achyranthes aspera L., Plantago major L., Santalum album L., Grewia asiatica L., and Coccinia grandis (L.) Voigt. Information on indigenous use of plants has led to discovery of many medicines

in use today. Scientific studies conducted on the above plants may lead to discovery of more effective drugs than in use at present.

Key Words: HAV/HBV/HCV infections, Plants, Bangladesh.

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COMBINATION THERAPY WITH IL-13 RECEPTOR ALPHA 2 DNA VACCINE AND AN IMMUNOTOXIN INHIBITS METASTATIC MURINE CANCERS

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Optimum efficacy of therapeutic cancer vaccines may require combinations that not only generate effective antitumor immune responses but also overcome immune evasion mechanisms mediated by progressing tumor. Previous studies have shown that interleukin-13 receptor α2 chain (IL-13Rα2), a unique tumor-associated antigen, is a promising target for cancer immunotherapy as high levels of IL-13Rα2 are expressed on a variety of human tumors, but not in normal tissues. IL13-PE, a targeted cytotoxin composed of IL-13 and mutated form of Pseudomonas exotoxin, induces specific killing of IL-13Rα2 positive tumor cells. Here, we demonstrate that combination therapy with IL13-PE and IL-13Rα2 DNA vaccine can cause a remarkable antitumor effect in advanced tumor metastasis models induced by 4T1 breast carcinoma and MCA304 sarcoma. Murine metastatic tumor models were developed by i.v. injection of either 1×10^5 4T1 or 1×10^6 MCA304 cells. Mice with established 4T1 metastasis received twice daily i.p. injections of IL13-PE on five consecutive days beginning day 7 followed by i.m. DNA vaccination on day 18, 23, 28 and 33. The combination therapy significantly ($P < 0.01$) prolonged overall survival of animals compared to control, IL-13PE alone or DNA vaccine alone. Similar results were observed in MCA304 sarcoma tumor model. Combination therapy treated mice showed higher CTL activity and IFN-γ release from splenocytes in vitro compared to the controls in both tumor models. Immunofluorescence analyses exhibited infiltration of $CD4^+$ and $CD8^+$ T cells in lung tissues of immunized mice. In addition, immunized mice recruited much lower number of myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs) and Regulatory T cells (Tregs) in lungs than PBS treated controls. Interestingly, flow cytometric analysis of splenocytes derived from 4T1 tumor bearing mice showed that MDSCs ($CD11b^+$/ $Gr\text{-}1^+$) express high levels of IL-13Rα2 compared to $CD11b^-$/ $Gr\text{-}1^-$ cells. These MDSCs were highly sensitive to IL13-PE in a concentration-dependent manner (IC50 = 80 ng/ mL) in protein synthesis inhibition assay, however, $CD11b^-$/ $Gr\text{-}1^-$ cells with undetectable level of IL-13Rα2 were not. Our results indicate that the mechanism of antitumor effect involved not only direct killing of tumor cells and cell mediated immune responses but also direct elimination of immunosuppressive elements, especially MDSCs and consequently TAMs and Tregs. These results provide first evidence that IL13-PE can eliminate MDSCs directly and a strong rationale for combining immunotoxins with cancer vaccines for the treatment of patients with advanced cancer.

Key Words: Cancer vaccine, Combination immunotherapy, Myeloid derived suppressor cell.

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INTRAVAGINAL TLR AGONISTS INCREASE LOCAL VACCINE-SPECIFIC CD8 T-CELLS AND HUMAN PAPILLOMAVIRUS-ASSOCIATED GENITAL-TUMOR REGRESSION IN MICE

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Cervical cancer is the second leading cause of cancer death in women worldwide, and the central etiologic agents are human papillomaviruses (HPV). HPV E6 and/or E7 oncogene-specific therapeutic vaccines are under active development, but have had limited clinical efficacy to date. Here we report that intravaginal (ivag) administration of CpG-ODN (CpG, a TLR9 agonist) or poly (I:C) (PIC, a TLR3 agonist) were able to increase approximately 5-fold the number of vaccine-specific IFN-gamma secreting CD8 T cells in the genital mucosa of mice after s.c. E7 vaccination, without affecting the E7-specific systemic response. Ivag CpG or PIC locally increased both E7-specific and total CD8 T cells, but not CD4 T cells, most probably through recruitment from the periphery. This previously unreported recruitment of activated CD8 T cells by ivag CpG or PIC was mediated by TLR9 and TLR3/Mda5 signaling pathways, respectively. Most interestingly, ivag CpG after s.c. E7 vaccination of mice bearing large established E7-expressing genital tumors resulted in more efficient tumor regression than vaccination alone. Antibody-mediated cell-specific depletion confirmed that this enhanced tumor regression upon ivag CpG was mediated by CD8 T cells. These findings suggest that topical application of immunostimulatory molecules might substantially increase the effectiveness of parenterally administered vaccines in reducing HPV-induced genital lesions in women.

Key Words: genital tumors, topical immunostimulation, therapeutic vaccine.

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ADENOVIRUS VECTOR-SPECIFIC CELLULAR IMMUNE RESPONSE INDUCED BY TRANSDUCED HUMAN DENDRITIC CELLS

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Recombinant Adenovirus (AdV) has been used in a wide range of gene transfer applications, including cancer immunotherapy and infectious diseases. The immune response to the Adenoviral vector is known to include a potent humoral response, resulting in generation

of circulating neutralizing antibodies. The role of the cellular response to AdV is poorly characterized. In the present study we evaluated the virus specific response to an E1/E3-deleted AdV encoding three melanoma tumor antigens: Tyrosinase, MART-1 and MAGE-A6. Despite a lack of Coxackie-Adenovirus Receptor expression by human Dendritic Cells (DC), these cells can be efficiently transduced by AdV. We performed weekly in vitro stimulations of healthy donor PBMC stimulated by AdV transduced human DC. CD4+ and CD8+ T cell responses were followed by multicytokine enzyme-linked immunosorbent spot (ELISPOT) assay to Interferon (IFN)-gamma, IL-2, IL-10 and TNF-alpha. Responses to the immunodominant AdV specific Hexon protein or to the entire AdV were determined. We found a strong Th1 focused response with INF-γ secreted by CD4+ cells beginning within 7 days of stimulation and carrying through all three stimulations both to hexon proteins and to the complete virus. Other Th1 cytokines were also secreted, mainly IL-2 and less so TNF-alpha. The hexon-specific CD8+ response was not reliably detected, but strong CD8+ T cell immunity was seen to the complete virus. This study provides insights into the cellular immune response to the AdV and will allow us to better harness these Th1 virus specific responses in melanoma vaccine trials.

Key Words: Cancer vaccine, Cellular immunity, Cytokine.

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ANTI-TUMOR ROLE OF INTERFERON-GAMMA PRODUCING CD1d-RESTRICTED NKT CELLS IN MURINE MALIGNANT PLEURAL MESOTHELIOMA

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Introduction: CD1d-restricted invariant NKT (iNKT) cells can provide adjuvant activity against cancer by producing large amounts of IFN-gamma which activate other immune cells, and orchestrate protective anti-tumor immunity. Recently, induction of the iNKT cell-dependent anti-tumor immune response using its ligand alpha-galactosylceramide (αGalCer) has been attempted in several tumor types. However, the role of iNKT cells in the tumor microenvironment has not yet been fully addressed. Our aim is to elucidate the role of iNKT cells in the thoracic cavity by using a murine malignant pleural mesothelioma (MPM) model.

Methods: Half a million of AB12 murine malignant mesothelioma cells were injected into the right pleural cavity of female Balb/c mice. Mice were sacrificed and the pleural effusion and tumors were collected on days 0 (before tumor cell injection), 7 and 14 after tumor cell injection. Lymphocytes were isolated and iNKT cells were identified using CD3 antibody and CD1d tetramers loaded with αGalCer. Cytokine expression was analyzed by intracellular cytokine staining using flow cytometry. To block iNKT cell activation, mice were treated with 0.5mg of anti-CD1d-blocking antibody 1 day before and 7 days after tumor cell injection. To activate iNKT cells, αGarCel was injected intraperitoneally on day 1, 5 and 9 after the tumor cell injection and the survival, the amount of pleural effusion on day 14 were analyzed.

Results: The ratio of iNKT cells to CD3 positive cells in the pleural cavity significantly increased after the tumor cell injection (day 0: 0.8±0.2%, day 7: 7.8±0.6%, day 14: 11.7±0.6%) while that in spleen did not change. The ratio of iNKT cells to CD3 positive cells in the tumor on day14 was 8.9±1.0%. These iNKT cells in the pleural cavity and the tumor showed higher CD25 expression compared with that in the spleen. The ratio of IFN-gamma positive iNKT cells in the pleural cavity was significantly increased after the tumor cell injection (day 0: 3.0±0.9%, day 7: 11.4±1.3%, day 14: 46.3±5.4%). The mice treated with CD1d-antibody showed less CD25 expression on iNKT cells, less IFN-gamma positive iNKT cells in the pleural cavity and increased amount of pleural effusion on day 14 compared with the control mice (1.1±0.1ml and 0.7±0.1ml, respectively, $p=0.045$). The mice treated with αGalCer showed significantly prolonged survival compared with the control mice (median survival time, 23.0 days and 17.0 days, respectively, $p < 0.0001$).

Conclusion: iNKT ells seemed to contribute to the anti-tumor immune response in murine MPM. Modulation of iNKT cells could be a new therapeutic approach for the patients with MPM.

Key Words: Animal model, Innate immunity, Tumor microenvironment.

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ELEVATED SERUM SOLUBLE CD40 LIGAND IN CANCER PATIENTS MAY PLAY AN IMMUNOSUPPRESSIVE ROLE

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Tumor cells are able to induce various cytokines and soluble receptors that have a suppressive effect on the immune system. In this study, we found that an extracellular portion of a membrane-bound ligand of CD40 (sCD40L), which could potentially be shed from activated platelets or lymphocytes, was significantly elevated in the serum of cancer patients compared to healthy donors. Peripheral blood mononuclear cells (PBMCs) from cancer patients had a relatively larger population of myeloid derived suppressor cells (MDSCs), defined as CD33+HLA-DR- cells, which express higher levels of CD40 than PBMCs from healthy individuals. T-cell proliferation and IFN-γ production decreased when these T cells were co-cultured with an increased amount of autologous CD33+ HLA-DR- cells. The addition of sCD40L had an additive inhibitory effect. PBMCs cultured in vitro with sCD40L also showed an expansion of CD4+CD25highFoxp3+ cells. Interestingly, sCD40L-induced enrichment of PD-1-expressing T cells was more dramatic in cancer patients than in healthy donors. We also reproduced data showing that pre-existing sCD40L inhibited IL-12 production from

monocytes upon activation with IFN-g plus LPS. These data suggest that the higher serum levels of sCD40L seen in cancer patients may have an immunosuppressive effect.

Key Words: Cellular immunity, Myeloid derived suppressor cell, PD-1.

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INTERVENTION OF MULTIPLE REGULATORY RECEPTORS INHIBITS Bim-MEDIATED T CELL DELETION AND PROMOTES DURABLE ADOPTIVE IMMUNOTHERAPY FOR CANCER

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Adoptive T cell immunotherapy requires that tumor-reactive T cells survive and be present in sufficient numbers to overcome an established tumor. However, tolerizing influences within the host or the tumor microenvironment can lead to deletion of transferred T cells, and inhibition of cytolytic effector function. The difficulty in maintaining a large population of infused tumor-reactive T cells represents a substantial challenge to the success of adoptive T cell immunotherapy, as the the multitude of positive and negative signals that determine T cell fate (i.e. survival, deletion, or anergy) remains enigmatic. Signals initiated downstream of the negative co-stimulatory molecules CTLA4, PD-1 or LAG3 typically serve to dampen T cell activation and function by interfering with stimulatory pathways. Using a well-established mouse model of CD8+ T cell tolerance, we have described novel and distinct roles for these inhibitory receptors in regulating T cell deletion and differentiation during adoptive immunotherapy for established cancer. Inhibiting ligation of CTLA4 and PD-1 in vivo together promoted persistence of transferred T cells despite powerful deletional signals that induced apoptosis through Bim-dependent mechanisms. Additional blockade of LAG3 was required for acquisition of full cytolytic activity by surviving T cells, and together these interventions provided durable immunotherapy for disseminated and progressive leukemia.

Key Words: Adoptive therapy, Cellular immunity, Combination immunotherapy.

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COMBINING IMMUNE THERAPY AND CHEMOTHERAPY AGAINST CANCER: ADRIAMYCIN SENSITIZES HUMAN TUMORS TO NK AND T CELL MEDIATED KILLING VIA AUGMENTED TRAIL SIGNALING

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Adoptive transfer of tumor-specific T cells or natural killer (NK) cells is being increasingly used in patients with cancer. The chemotherapy agent adriamycin (ADR) has previously been shown to sensitize tumors to lysis by tumor infiltrating lymphocytes (TIL). Here, we investigate the underlying mechanisms of sensitization by ADR to TIL-mediated killing and also examine whether ADR-treated tumors become sensitive to killing by NK cells. Compared to untreated tumors an 8-fold and a 3-fold increased killing of ADR-treated tumors was observed by melanoma-antigen specific TILs ($p<0.05$) and NK cells ($p<0.01$) respectively (figure 1). We did not observe any differences in surface expression of TRAIL-receptors on ADR treated tumors. However, in presence of neutralizing antibodies to TRAIL, but not Fas ligand nor perforin inhibitors, both NK cell ($p<0.01$) and TIL ($p<0.05$) mediated killing of ADR-treated tumors was significantly reduced. Exposure of human tumors to low doses of ADR resulted in decreased expression of the caspase-8 inhibitory protein cFLIP. Consequently, increased activity of caspase-8 was observed in ADR treated tumors upon co-culture with NK or T cells. In addition, western blot analysis revealed an increased Bid and caspase-9 cleavage in ADR-treated tumors when cultured with NK cells or TILs, demonstrating the involvement of the mitochondrial apoptotic pathway. Also, NK cell-mediated killing of ADR-sensitized tumors was significantly decreased in presence of caspase inhibitors. In summary, these results show that ADR sensitizes human tumors to killing by NK and T cells via increased TRAIL receptor signaling and initiation of both intrinsic and extrinsic apoptotic pathways. Our findings encourage using ADR as pre-treatment in patients with cancer, receiving adoptive cell therapy with either TILs or NK cells.

Key Words: Adoptive therapy, Chemotherapy, Combination immunotherapy.

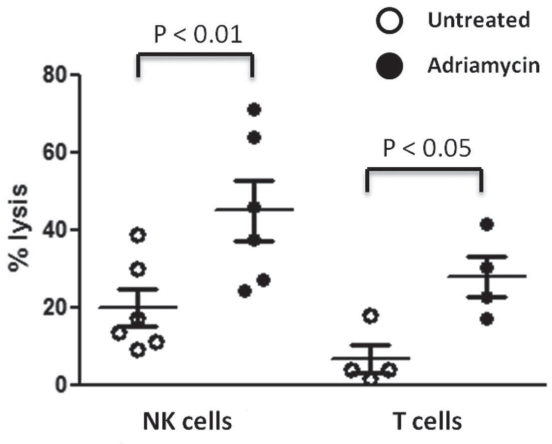

Figure 1. *NK and T cell cytotoxicity against untreated or ADR treated human bladder cancer cell line (J82). E:T ratio = 1:1.*

Presenting author underlined. *Primary author in italics.*

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PROCLAIM℠: A PROSPECTIVE OBSERVATIONAL REGISTRY OF INTERLEUKIN-2 IN CANCER

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Interleukin-2 (IL-2) is a pleiotropic cytokine that impacts the function of receptor-bearing immune cells, and was FDA approved in 1990s based on multiple Phase 2 clinical trials that demonstrated its ability to induce durable responses in patients with metastatic melanoma and renal cell carcinoma. Despite this notable attribute, the application of IL-2 has been limited by its toxicity to selected patients in specialized units. Recent events have challenged this perception. Over the last 20 years, clinical trial data suggests that changes in patient screening and IL-2 toxicity management algorithms may have led to improved patient outcomes and safety. The wide spectrum of IL-2 doses, schedules, regimens, routes of administration and combination with other agents complicates the modern real-world utilization of IL-2. Thus, the modern safety and efficacy profile of IL-2 in large numbers of patients outside of clinical trials remains unknown. The advent of new therapeutic approaches such as mutant B-Raf inhibition and CTLA4 blockade further highlights the necessity to investigate these issues on an ongoing basis.

The **Pr**oleukin® **O**bservational Registry to Evaluate the Treatment Patterns and **Cl**inic**a**l Response **i**n **M**alignancy (PROCLAIM℠) is aimed at addressing this critical knowledge gap. After validation of the methodology and training set, this registry will prospectively track the use of IL-2 in patients with a diagnosis of malignancy. This US-based observational database will be established to provide a source of information to answer investigator queries such as the mortality rate and safety of IL-2 administration, response rate, response characteristics, and durability of response in a large number of patients in the context of current practice. This registry will also collect the dose, schedule, regimen, and supportive measures surrounding IL-2 administration. The output from this registry will be summarized on a yearly basis. In addition, the database will be used to generate and prospectively test hypotheses about patient selection, sequencing of therapy, and optimal IL-2 schedule and dose.

The PROCLAIM registry may help to improve our understanding of the proper applications of this important therapy, especially in the context of rapid evolution of new therapeutic paradigms emphasizing the cure of solid tumors.

Key Words: Interleukin-2, Melanoma, Renal cell carcinoma.

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DOSE ESCALATION OF ADJUVANT CAP1-6D/MONTANIDE/GM-CSF VACCINATION IN HIGH RISK PANCREATIC CANCER: INDUCTION OF CEA-SPECIFIC CD8+ T CELL RESPONSES AND EARLY SUGGESTION OF CLINICAL BENEFIT

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In a randomized phase II trial, HLA-A2+ pancreatic cancer (PC) patients with CEA+ tumors were immunized with a modified CEA (CAP1-6D) peptide/Montanide/GM-CSF vaccine at two-week intervals for maximum one year or until disease progression. Because the optimal dose of peptide for vaccination is not clear, cohorts of patients were randomized to receive 0.01, 0.1, or 1.0 mg of peptide per vaccination. Heparinized blood was drawn before treatment, monthly during the vaccination, and at the end of study. PBMCs were isolated and cryopreserved for immune assays. CEA-specific CD8+ CTL responses were tested by IFN-γ ELISPOT, against both the substituted and the wild-type peptides. Poor CEA-specific CD8+ CTL responses were detected by a direct ex vivo assay, so a short in vitro expansion was performed. Briefly, purified CD8+ T cells were stimulated with irradiated peptide-pulsed CD8- cells and β2-microglobulin, along with IL-2 (10 U/ml). Expanded CD8+ T cells were re-stimulated with peptide-loaded T2 cells for IFN-γ ELISPOT analysis. We found that reproducible induction of T cell responses did not occur until the highest dose level of peptide used to vaccinate, at which 5/5 patients showed induction of immunity. T cell responses reacted to both the substituted and the wild-type CEA peptides. Remarkably, one patient with locally advanced unresectable PC showed an unexpectedly long period of stable disease (> 4 years) after receiving highest dose vaccination. We conclude that the CAP1-6D peptide is immunogenic in this patient population, that high quantities of CEA peptide in the vaccine preparation are necessary for induction of strong T cell responses, and that reactivity with the native CEA peptide is also induced. The prolonged disease control in a subset of patients warrants further development of immunotherapy approaches in this patient population.

Key Words: Cancer vaccine, CD8+ T cells.

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ROLE OF NK AND NKT CELLS IN BREAST CANCER PATIENTS: ASSOCIATION WITH FAVORABLE PROGNOSIS

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Among cells of the innate immune system NK and NKT cells are known to be responsible for tumor immune surveillance. Recognition of tumor cells by NK cells is mediated by the interaction of activating and inhibitory receptors with ligands expressed on the target cells. NK cells also express adhesion molecules, thereby interacting with tumor cells and dendritic cells. NKT cells recognize glycopeptides expressed on MHC class I-like molecules, and play a key role in cross talk between innate and adaptive immune responses.

In the current study we analyzed the differential expression profile of the NK activatory and inhibitory receptors, gene involved in NK cell-target interactions, as well as NKT signaling pathways in breast cancer patients whose tumors relapsed vs. those who remained relapse-free.

Gene expression analysis was performed on tumor specimens with at least 10% infiltrating cells deriving from 9 patients with up to a 7-year relapse-free survival and 8 patients who developed tumor relapse within 1-4 years after the treatment. Data were analyzed by BRB tools and Partek software.

Our results showed that patients with a favorable prognosis showed increased expression of genes involved in NK-mediated anti-tumor responses as well as genes involved in NK- NKT signaling pathways. These included up-regulation of leukocyte function-associated antigen 1 (LFA-1), CD96 and CRTAM .Our observation suggests that the NK and NKT signatures are associated with favorable outcome in breast cancer patients. It is to be determined whether signatures of NKT cells and NK cells may predict conversion of myeloid-derived suppressor cells (MDSC) into dendritic cells and in turn release of T cells from MDSC-mediated suppression.

Key Words: Breast cancer, Innate immunity, NK cells.

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GENOTYPIC, PHENOTYPIC AND FUNCTIONAL ANALYSIS OF MELANOMA

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Background: It is assumed that transcriptional signatures displaying a status of immune activation of cancer reflect genes expressed by infiltrating immune cells. However, it is possible that part of the immune signatures is due to constitutive activation of cancer cells. The best evidence supporting this phenomenon is the constitutive activation of immune genes by pure cancer cell cultures in vitro. At present, no correlative study that tested whether the signatures observed in tumors are also present in cell lines derived from the same tumors.

The present study focused on the constitutive activation by phosphorylation of STAT proteins (pSTAT) in cancer cells and their relationship with immune activation in the parental tumors; in particular the balance between the anti-apoptotic and regulatory effects of pSTAT-3 was compared to the pro-apoptotic and pro-inflammatory activity of pSTAT1.

Methods: Constitutive levels of intracellular STATs and their relative activation (pSTAT) (STAT1,STAT2, STAT3, STAT4, STAT5, STAT6) was assessed by flow cytometry analysis and proteomic profiling in 15 melanoma cell lines; this information was correlated with transcriptional patterns observed in the cell lines as well as the parental tissues based on Affymetrix Human gene 1.0 ST arrays.

Results: According to the different intracellular level of pSTAT1 and pSTAT3 proteins, the cell lines were classified in 3 groups: pSTAT1 positive (7 cell lines), pSTAT3 positive (4 cell lines) and Null (4 cell lines). Class comparison (Student's T-test $p2<0.05$) identified genes differentially expressed by pSTAT1 compared to pSTAT3 cell lines with up regulation of CCL5 and TAP-1 by pSTAT1 positive cells and LIFR by pSTAT3 positive cells. Comparison, of the same signature gene expression in tissues approximated the results observed in cell lines suggesting that part of the profile observed in vivo is related to the intrinsic biology of cancer cells rather than being limited to the activation of infiltrating immune cells

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

Key Words: Chemokines, Innate immunity, Melanoma.

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SYSTEMIC CONCANAVALIN A TREATMENT REVERSES THE SUPPRESSIVE FUNCTION OF HEPATIC CD11B+GR-1+ MDSCS IN TUMOR-BEARING MICE

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Background: Myeloid Derived Suppressor Cells (MDSCs) represent a heterogeneous population of myeloid cells at different stages of development. Their numbers increase in the spleen, blood, liver and tumor of mice upon tumor growth. Murine MDSCs co-express Gr-1 and CD11b and has been shown to impair immune responses. Therefore, MDSCs are one of the potent mediators of cancer dependent immune suppression.

The aim of this study was to examine the function of liver MDSCs in tumor-bearing (TB) mice after administration of Concanavalin A (ConA).

Methods: Female 8-10weeks old C57BL/6 mice were used. 10'6 EL-4 cells were injected subcutaneously. Tumor-free (TF) served as controls. Hepatitis was induced by intravenous administration of Concanavalin A. Alanin-aminotransferase (ALT) and aspartate-aminotransferase (AST) were measured in serum and histological examination of liver sections were performed to grade liver inflammation. Phenotype of infiltrating cells and ROS production were analysed by FACS. Suppressive function of MDSCs was studied using ex vivo co-cultures.

Results: ConA injection induced higher serum ALT/AST concentrations and more CD11b+Gr-1+ cells in spleen, blood and liver and more severe hepatitis in TB mice then in TF controls. CD11b+Gr-1+ cells from ConA-treated TB and TF mice produced similar amount of ROS. However MDSCs from TB mouse liver lost their ability to suppress T-cell responses in vitro.

Conclusion: Our data demonstrates that TB mice are more susceptible to ConA-induced hepatitis than TF controls. TB mice developed more severe inflammation upon ConA injection then TF mice. Currently we investigate how ConA can switch CD11b+Gr-1+ cells from a suppressive to a pro-inflammatory phenotype.

Key Words: Innate immunity, Myeloid derived suppressor cell, Tumor microenvironment.

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ANALYSIS OF NF-KB AND IRF-1 NUCLEAR TRANSLOCATION AND CO-LOCALIZATION IN 15 MELANOMA CELL LINES: A CELL HETEROGENEITY ISSUE

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NF-kB is commonly constitutively activated in a wide variety of solid tumors such as melanomas and it is connected to tumorigenesis because of its anti-apoptotic properties. As inflammation is believed to foster cancer growth, NF-kB activation with its combined pro-inflammatory and anti-apoptotic activities is a theoretical hub of tumorigenesis. Several studies, however, observed a cancer phenotype where inflammation bears good prognostic connotation and predicts better responsiveness to therapy. In particular, constitutive activation of the interferon (IFN)-γ/STAT-1/interferon regulatory factor (IRF)-1 axis (Th1 phenotype) was identified as a good predictor in several solid tumors. Although IRF-1 over expression is associated with better prognosis and likelihood of response to therapy, such correlation is not absolute, suggesting that other factors are involved in the process. IRF-1 (a pro-apoptotic transcription factor) is known to enhance NF-kB pro-inflammatory activity while simultaneously blocking its anti-apoptotic effects. Thus, we hypothesize that the coordinate activation of the two transcription factors is more likely to influence the phenotype of melanoma than each one of them separately. Here we study the nuclear translocation, co-distribution and co-localization of IRF-1 and NF-kB (RelA subunit) in 15 immunologically active melanoma cell lines.

15 melanoma cell lines were cultured and probed for IRF-1 and NF-kB (p65) staining. IRF-1 and NF-kB nuclear translocation and their co-localization were assessed by using the Multispectral Imaging Flow Cytometry (Amnis ImageStream).

Accordingly with the literature, we found a significant positive correlation between IRF-1 and NF-kB nuclear translocation among the 15 melanoma cell lines ($R=0.6416$, $p=0.0074$), indicating a combined role of these factors in immunologically active melanoma cells. However the percentage of IRF-1 and NF-kB nuclear co-localization resulted to be low and highly variable in all the cell lines analyzed. This could be due to the different behavior of cell sub-populations within the same cell line.

This study shows the combined importance of IRF-1 and NF-kB in immunologically active melanoma cell lines. However the IRF-1 and NF-kB co-localization will be further assessed after cell cycle synchronization, in order to avoid bias due to the heterogeneity of each cell line.

Key Words: Melanoma.

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ZOLEDRONIC ACID TREATED MONOCYTES ENHANCE TRAIL-MEDIATED ANTI-TUMOR EFFECT OF NK CELLS

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Natural killer (NK) cells are innate lymphocytes able to kill tumor cells through ligation of receptors to TNF-related apoptosis-inducing ligand (TRAIL). However, peripheral blood non-activated NK cells express low levels of TRAIL and are unable to kill TRAIL-receptor positive tumors. Zoledronic acid (ZA) is a bisphosphonate known to up-regulate expression of soluble TRAIL on human gammadelta T cells. Here we investigated the functional consequences including expression of TRAIL of ZA treatment on human NK cells. Upon treatment of purified NK cells, ZA had no effect on the viability, proliferation or expression of TRAIL or any other surface molecules analyzed including, NKG2D, Fas ligand, NKp44, NKp46, perforin or granzyme B. However, in presence of interleukin-2 and autologous irradiated monocytes, a five-fold increase in expression of TRAIL was observed on NK cells ($p=0.033$) compared to co-cultures not treated with ZA. In contrast, TRAIL expression on NK cells did not increase in presence of ZA following co-culture with irradiated CD3+ T cells. Consequently, NK cells co-cultured with ZA-treated monocytes efficiently killed tumor cells rendered sensitive to TRAIL-mediated killing by pre-treatment with the proteasome inhibitors bortezomib or bAP-15. The percent tumor cell lysis by ZA-treated NK cell co-cultures increased 1.5- to 4-folds compared to untreated NK cell co-cultures ($p=0.04$). Neutralizing antibodies to TRAIL reduced the cytotoxicity demonstrating the increased killing was mediated via TRAIL. In tumor-bearing SCID-beige mice, adoptive infusion of ZA-treated NK cells resulted in significantly delayed tumor progression compared to mice infused with untreated NK cells ($p=0.03$). We demonstrate that ZA-treated monocytes can up-regulate the expression of TRAIL on NK cells resulting in significantly augmented killing of TRAIL sensitive tumor cells in vitro and in vivo. We also show that the proteasome inhibitors bortezomib and bAP-15 can restore sensitivity to TRAIL-mediated killing of TRAIL-resistant tumor cells. These studies have implications for the design of adoptive cell therapy protocols with NK cells in patients with cancer.

Key Words: Adoptive therapy, Innate immunity, NK cells.

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PROSTATE CANCER SPONTANEOUSLY INDUCES T CELL RESPONSE TO PROMOTE ITS PROGRESSION

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Cancers often induce antigen-specific T cell responses. However, the role of these tumor-activated T lymphocytes in regulating pro-tumor versus anti-tumor immune response remains controversial. We report that lymphocytes are essential for tumor progression in an autochthonous prostate cancer model. Activated T lymphocytes gradually accumulate inside the tumor during the process of de novo spontaneous tumor development driven by the tissue-specific expression of an oncogene. The tumor-infiltrating T lymphocytes express membrane lymphotoxin (LT), and the signaling through LTβ receptor (LTβR) is essential for the recruitment of CD11b+Gr-1-F4/80+ macrophages inside the tumor and the tumor development. Intriguingly, blockade of high mobility group box 1 (HMGB1) drastically inhibits tumor-antigen-specific T cell activation, infiltration and tumor progression. Our study has demonstrated that "endogenous danger signal" HMGB1 produced by a growing tumor is essential to bridge innate and adaptive immune responses, which could further promote tumor progression over time in an LTβR-dependent manner. Our finding may have important implications for future prostate cancer prevention and therapy.

Key Words: Cellular immunity, Prostate cancer, Tumor infiltration lymphocytes.

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HARNESSING MERKEL CELL POLYOMAVIRUS-SPECIFIC IMMUNE RESPONSES TO TRACK AND TREAT MERKEL CELL CARCINOMA

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Merkel cell carcinoma (MCC) is an aggressive skin cancer with few treatment options for progressive disease. Merkel cell polyomavirus (MCPyV) integrates into most MCCs and encodes truncated T-antigen (T-Ag) oncoproteins required for tumor growth. We have recently reported that 1) anti-T-Ag antibodies dynamically reflect clinical tumor burden, 2) intratumoral CD8 T cells predict survival for MCC patients, and 3) MCPyV-specific CD8 and CD4 T cells are present in MCC tumors and blood. Application of these concepts to one of our patients (w447) allowed us to detect occult disease and generate adoptive immune therapy.

Patient w447 presented with a primary MCC tumor on his left hip. Tumor infiltrating lymphocytes (TIL) were expanded in the absence of exogenous antigen and assessed for interferon-gamma production following exposure to peptide pools derived from MCPyV T antigen. CD8 T cells specific for an MCPyV peptide, EWWRSGGFSF (EWW), were identified. This epitope was restricted to HLA-A*2402. An MCPyV HLA-A24/EWW tetramer was generated, and EWW-specific T cells were detected in the blood of 3 of 6 HLA-matched MCC patients.

Increasing anti-T-Ag antibody titers suggested disease relapse in w447, who remained asymptomatic. Indeed, a PET scan detected an FDG avid nodule near the head of the pancreas. There are two likely explanations for disease progression despite the presence of T cells specific for MCPyV T-Ag oncoproteins, both of which are amenable to therapeutic reversal. First, as in the majority of MCC tumors, w447 tumor expressed low levels of HLA class-I required for CD8 T cell recognition. We show that a single administration of either XRT (2-8 Gy) or any of several interferons to can significantly increase HLA-I expression in an MCC cell line that was HLA-I-negative and MCPyV-positive. Second, unlike expanded TIL, MCPyV-specific T cells from blood failed to produce IFN-γ when challenged with peptide directly ex vivo. Preliminary results indicate that these T cells had increased PD-1 and CTLA-4 expression compared to CD8 T cells of other specificities, suggesting T cell exhaustion. Inhibitors of such immune-regulatory receptors are promising agents that may overcome T cell dysfunction in MCC.

In September, we plan to administer autologous HLA-A24-restricted EWW-specific T cells following HLA-I-enhancing therapy to patient w447. Data on T cell persistence, phenotype, functional characteristics, and potential efficacy of adoptive immune therapy will be presented.

Key Words: Merkel cell carcinoma, Merkel cell polyomavirus.

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PROGRESS IN THE CLINICAL DEVELOPMENT OF A NOVEL IL-12 DNA IMMUNOTHERAPEUTIC FOR THE TREATMENT OF ADVANCED PERITONEAL CARCINOMATOSIS OF OVARIAN AND COLORECTAL ORIGIN

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A novel DNA immunotherapeutic for local treatment of cancer is described. EGEN-001 is composed of an interleukin-12 (IL-12) gene expression plasmid and a biocompatible delivery lipopolymer, PEG-PEI-Cholesterol (PPC), assembled into well-characterized nanoparticles. EGEN-001 is designed to increase local concentrations of IL-12 and related cytokines at cancer site. In both human and animal studies, intraperitoneal treatment of EGEN-001 resulted in significant accumulation of plasmid DNA and immunocytokines in the peritoneal cavity but not as much in blood. The treatment did not result in systemic toxicity over several month of weekly treatment, a distinct safety advantage over current therapies. The safety and biological activity of EGEN-001 has also been demonstrated in combination with cytotoxic drugs both in ovarian cancer patients and in animal models of cancer. The common adverse events of EGEN-001 treatment were abdominal discomforts and low grade fever, which were generally reversible within 24 hours. In the combination trial, a small number of patients also experienced transient hypotension. In the monotherapy study, 69% of patients had progressive disease and 31% of patients had stable disease. The average overall survival at high EGEN-001 doses was ~2 years as compared to 8-10 months in this patient population. In the combination trial with paclitaxel/docetaxel, 38% of patients had complete or partial response, 15% of patients had stable disease, and 46% of patients had progressive disease. The CA-125 levels remained below treatment levels in 61% of patients at the 6-month follow-up. In the mouse model, a single dose of intraperitoneal EGNE-001, encoding mouse IL-12 gene, resulted in significant increases in mouse IL-12 and IFN-g levels in peritoneal fluid but several fold less in serum. EGEN-001 treatment also produced significant increases in Th1 transcripts in cancer bearing mice. In ovarian cancer patients, EGEN-001 treatment administered alone or in combination with chemotherapy produced significant increases in cytokine levels in peritoneal fluid but several fold less in serum. A Phase II trial of EGEN-001 for the treatment of peritoneal carcinomatosis in platinum resistant ovarian cancer patients is in accrual stage. The anti-cancer activity of EGEN-001 has also been demonstrated in mice with peritoneally disseminated or subcutaneously implanted colon cancer. A Phase I/II trial of EGEN-001 in patients with peritoneal carcinomatosis of colorectal cancer is in accrual stage. EGEN-001 is produced under good manufacturing practice with a process scalable to Phase III needs. The final product is a lyophilized material with more than 3 years of shelf-life at -20 C and -80 C.

Key Words: Combination immunotherapy, Cytokine, Targeted therapeutics.

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ADXS11-001 IMMUNOTHERAPY TARGETING HPV-E7: PRELIMINARY SAFETY DATA FROM TWO PHASE 2 STUDIES IN WOMEN WITH CIN 2/3 AND WITH RECURRENT/REFRACTORY CERVICAL CANCER

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ADXS11-001 is an attenuated live Listeria monocytogenes (Lm) immunotherapy currently in clinical trials for HPV-associated dysplasia and malignancy. The use of the Lm vector has distinct advantages in immunotherapy. Being a gram positive intracellular bacterium, the vector serves as its own adjuvant and infects the APC where it naturally cross presents, stimulating both MHC class 1 and 2 pathways. The vector also mobilizes myeloid precursors, upregulates chemokines, and inhibits Treg and MDSC activity within the lesion but not elsewhere. The HPV E7 fusion protein-directed cytotoxic T-cell response targets HPV-E7 transformed cells and results in an increased ratio of antigen-specific cytotoxic T-cells to Treg cells infiltrating the lesion reversing immunosuppression.

Here we describe the incidence rate and pattern of adverse events associated with ADXS11-001 administration in 2 ongoing Phase 2 clinical trials in women with early or very advanced HPV-driven neoplasias. In Lm-LLO-E7-015, women with progressive cervical cancer who have failed cytotoxic therapy are randomized to 3 doses of ADXS-011 at $1x10^9$ CFU or 4 doses of $1x10^9$ CFU with cisplatin chemotherapy between doses 1 and 2. As of 7/15/11 52 subjects have received 112 doses. Lm-LLO-E7-07 is a randomized, single blind, placebo controlled, dose escalation study in subjects with CIN 2/3. The initial 40 subject cohort received 3 doses each of $5x10^7$ CFU or placebo (3:1 randomization). As of 7/15/11 30 subjects have received 75 doses. In both studies naprosyn and oral promethazine are given as premedications and a course of ampicillin is given 3 days after infusion as a precautionary measure.

From this clinical experience, a clear pattern of treatment-related adverse events has emerged consisting of fever, chills, nausea and vomiting which are consistent with the release of immunologic cytokines commonly associated with immune activation. Between 15-23% of the doses administered have been associated with a drug-related adverse event; typically a transient Grade 1 or 2 (mild-moderate) flu-like symptom, which appears within a few hours to 3 days after infusion. Symptoms either self-resolve or respond quickly to symptomatic treatment. Thus far, there have been no serious adverse events associated with ADXS11-001 in 187 doses, no evidence of listeriosis, no persistent symptoms, no delayed symptoms, and no evidence of cumulative toxicity in subsequent doses.

This immunotherapy can be safely administered to healthy young subjects as well as patients with advanced cancer and presents a predictable and manageable safety profile. Updated findings will be presented at the meeting.

Key Words: Active immunotherapy, Cancer vaccine, Cellular immunity.

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CIMAVAXEGF® CANCER VACCINE FOR NON SMALL CELL LUNG CANCER (NSCLC) THERAPY: PARTIAL REPORT OF A PHASE III CLINICAL TRIAL

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The Epidermal Growth Factor Receptor (EGFR) is a well recognized oncogen over-expressed in several malignancies of epithelial origin. Several therapeutic approaches targeting this receptor are being developed and tested in the clinical setting. The more known are monoclonal antibodies that recognize the EGFR and small molecules such as tyrosine kinase inhibitors. Our approach focuses the active immunotherapy using the EGF as antigen to develop an immune response that captures the circulating EGF and circumventing its binding to the EGFR.

Clinical testing with the EGF vaccine has began in 1995. During this clinical experience more than 1500 patients have been vaccinated with CIMAvax EGF, displaying a safety profile and immunogenicity. It has shown a clinical benefit through the improvement of the survival of vaccinated patients as compared to controls. The long term tolerability of this therapeutic approach has been also evidenced in five phases I/II and two phase II clinical trials.

Here we report the clinical results of the first 260 evaluable patients from the current ongoing Phase III clinical trial (3rd efficacy trial). According to its design, 579 patients with advanced (stages IIIb and IV) NSCLC, who respond to the 1st line chemotherapy, are stratified per age (younger than 60 years old and equal or older than 60 years old), in 2 groups of 198 and 381 patients respectively, and then randomized for vaccination or best supportive care in a 2:1 ratio (vaccine: control).

Vaccinated patients received the emulsified vaccine composed by EGF, P64k as carrier and Montanide ISA 51 VG as adjuvant, in 4 induction doses within 14 days intervals, and monthly re-immunization afterwards for all the follow-up period. Each vaccination consisted in 4 injections (2 deltoids and gluteus), with 0.6 mg of protein in each injection site (2.4 mg per immunization).

All vaccinated patients survived significantly more than controls, 12.4 vs. 8.8 months of median survival respectively, with significant survival rates from 12, 24 up to 36 months of follow up. The same clinical behaviour was observed in the strata corresponding to younger than 60 years old patients. Continuation of the trial is granted based in the effect of vaccination observed in these partial cut-off results.

Key Words: Cancer vaccine, Lung cancer, Phase III.

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CROSSTALK BETWEEN MELANOMA AND IMMUNE CELLS: ROLE OF JAK/STAT PATHWAY IN TUMOR CLINICAL RESPONSE

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We have recently summarized several observations describing a cancer phenotype where the expression of immune effector mechanisms reflect patterns commonly observed during the inflammatory response against pathogens, which leads to elimination of infected cells (Ascierto ML et al. J Transl Med, 2011). Several studies determined an association between this inflammatory status and a favorable natural history of the disease or a better responsiveness to cancer immune therapy. In the current study, using Affymetrix Human gene 1.0 ST arrays, we performed a prospective molecular profiling of melanoma metastases belonging to patients receiving adoptive therapy and adopted a low stringency gene enrichment strategy for class discovery/comparison (N=113). This approach segregated melanoma metastases into two distinct phenotypes according to the expression of genes involved in immune functions. Interestingly, an Interferon (IFN)-gamma type signature with enhancement of the Stat 1/2/3, IRF1, JAK and NF-KB pathways which correlated with the expression of genes involved in antigen processing and presentation, was found to be inversely correlated with the expression of the microphthalmia-associated transcription (MITF)-cluster of melanoma differentiation antigens and with cancer testis antigens. This inverse correlation could be ascribed to negative selection of cancer cells simultaneously expressing the antigens target of immune recognition and the corresponding antigen presenting molecules. Additionally, class comparison and leave one out cross validation between patients who did or did not respond to therapy identified signatures potentially predictive of overall response (OR). These results suggest that lesions from patients likely to respond to therapy are characterized by enhanced expression of genes related to interleukin-6 signaling and JAK/Stat Signaling. In particular, an up regulation of the receptor subunit Gp130, SHP2 as well as Janus kinases (Jak)-1 and -2 together with signal transducers and activators of transcription (STAT)-1, -2, 3, and -5 was found in responder patients compared to the non responder ones (Fisher test p value 0.0001). These results may be explained by redundant signaling by various cytokines and growth factors through engagement of cell surface receptors that lead to JAK-STAT pathway activation. Thus, differential expression or alterations in the regulation of JAK-STAT signaling in cancer cells could determine the differential responsiveness of such cells to therapeutic cytokines.

Key Words: Adoptive therapy, Melanoma.

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SEEN BUT UNSEEN: THE ELUSIVE IMMUNOGENICITY OF OVA-EXPRESSING TUMOR CELLS

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Background: Activated natural killer (A-NK) cells are able to efficiently lyse most cancer cells and may therefore provide dendritic cells with possibly antigenic material for presentation to T cells. Furthermore, cross-talk between activated NK cells and iDCs bias the downstream immune response in the Th1 direction. Thus, with the goal of inducing strong Th1-comitted adaptive anti-tumor immunity, we injected a mixture of highly cytotoxic NK cells and in vitro propagated immature DCs into sc tumors induced by OVA-expressing B16 tumor cells. Despite the fact that the B16-OVA cells are killed by specific CTLs recognizing OVA-derived SIINFEKL (OT-I cells), the NK/DC intratumoral injection did not lead to activation of naïve OT-I cells in the draining lymph nodes and did not result in reduction of tumor growth. We excluded NK mediated killing of the DCs as a reason for the lack of CTL priming, and hypothesized that the B16-OVA cells possibly were non-immunogenic despite their sensitivity to killing by OT-I CTLs.

Findings: A) OVA-expressing and wild-type tumor cells were stained with an antibody recognizing SIINFEKL-H-2Kb. EL4-OVA stained positive, but B16-OVA cells did not. Pretreatment with IFNg increased MHC expression, but only EL4-OVA showed increased staining with anti-SIINFEKL-H-2Kb antibody. B) In vitro stimulation of splenocytes from naïve OT-I animals with UV treated EL4-OVA cells and low dose IL-2 resulted in significant proliferation of the SIINFEKL-specific OT-I cells. No proliferation was seen when OT-I splenocytes were stimulated with wtEL-4, wtB16 or B16-OVA cells. C) Vaccines composed of ex vivo propagated DCs and killed B16-OVA were not able to prime naïve OT-I cells in vivo. However, addition of OVA protein or SIINFEKL peptide to the inoculum resulted in proliferation of OT-I cells in the draining LNs.

Conclusion: While tumor cells may produce tumor-specific peptides (as here; SIINFEKL), which are clearly recognizable by CTLs (here: primed OT-I), they may not provide the antigen in a form, amount or context needed to prime these T cells. We are now investigating whether manipulation of proteasome activity and autophagic flux may increase tumorigenicity.

Key Words: Animal model, Cancer vaccine, CD8+ T cells.

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META-ANALYSIS OF ACUTE ALLOGRAFT REJECTION GENE EXPRESSION PROFILING STUDIES REVEALS ANALOGIES BETWEEN PATHWAYS ACTIVATED DURING ALLOGRAFT AND TUMOR REJECTION

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Background. By studying the events in real-time that occur before and during therapy in tumor microenvironment, we previously showed that tumor rejection is associated with the activation of specific pathways. Based on studies conducted in humans, we recently hypothesized that different immune-mediated tissue destruction processes (i.e. cancer, infection, autoimmunity) share common convergent final mechanisms that lead to the activation of these pathways. We called this phenomenon the "Immunologic Constant of Rejection (ICR)." The elements of the ICR include molecular pathways that are consistently described through different immune-mediated tissue destruction processes and demonstrate the activation of interferon-stimulated genes (ISGs), the recruitment of cytotoxic immune cells (primarily through CXCR3/CCR5 ligand pathway), and the activation of immune effector function genes (IEF genes; granzymes A/B, perforin, etc.).

Here, we challenged the ICR hypothesis by systematically reviewing studies analyzing gene expression by microarray during acute allograft rejection. We found the pillars of the ICR consistently present among the studies reviewed, despite implicit heterogeneity.

Methods. Microarray studies providing original data and performed on human tissue biopsies during established acute allograft rejection were selected and evaluated.

The compiled list of key genes came from those reported as upregulated in the original publications, most of which were predominantly immune-related. In total, fifteen unique datasets met the search criteria. Of these datasets, three meta-analyses and one multi-dataset comparative analysis were among those selected for inclusion.

The Ingenuity Systems Pathway Analysis (IPA) (www.ingenuity.com) and MetaCore (www.genego.com) were used to illustrate the relationships among the compiled list of key genes.

Results. We found that ICR pathways activated during immune mediated tumor rejection are frequently activated during acute allograft rejection across studies conducted by different investigators in different organs (i.e., kidney, lung, heart and liver).

Conclusions. High-throughput gene expression profiling has emerged as a powerful and reliable tool in investigating immune response in vivo in human. The detection of these highly conserved pathways among different conditions suggests that they may have a central role in mediating immune mediated tissue destructions. Cancer immunotherapies aimed to trigger these key pathways should be investigated.

Key Words: Cell trafficking, Cellular immunity, Cytokine.

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ADDITION OF α-GALACTOSYLCERAMIDE TO TUMOR PULSED DENDRITIC CELL VACCINES ENHANCES TUMOR ANTIGEN-SPECIFIC T CELL RESPONSES AND LEADS TO CLINICAL RESPONSES IN PATIENTS WITH METASTATIC MELANOMA

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Metastatic melanoma can respond to a range of immune therapies but significant improvements in clinical outcomes are required. We aim to achieve this by combining methods to enhance tumour-antigen specific cytotoxic T cells (CTL) with those that enhance additional immune effector arms that may combat tumour resistance through a greater repertoire of anti-tumour mechanisms. Human invariant Va24Vb11 T cell receptor (TCR) expressing natural killer T cells (NKT cells) activated by a-Galactosylceramide (a-GalCer, KRN7000), presented by CD1d on antigen presenting cells, exert significant anti-tumour activity against a variety of malignancies in human in vitro models and in vivo in mice. Mechanisms of NKT mediated antitumour activity reported include direct cytotoxicity, killing of CD1d expressing tumor-associated macrophages, antiproliferative effects and secondary effects including activation of antigen specific T cells and NK cells. We conducted a Phase I trial evaluating the potential anti-tumour effects of dendritic cells co-pulsed with a-GalCer and peptide tumour antigens in patients with active metastatic melanoma. The goal of this study was to determine whether NKT cell activation can enhance stimulation of melanoma antigen-specific CTL in the clinical setting and, if so, what is the optimal administration route for this to occur. We also aimed to obtain preliminary clinical evidence for possible therapeutic effects of treatment with Mo-DC co-pulsed with a-GalCer and melanoma specific peptides. Our results show that, although IV administration produces the greatest activation of NKT and NK cells (and hence potential for anti-tumour activity), the ID route is more potent at increasing peripheral blood antigen specific CTL and had markedly better clinical outcomes. Patients treated with DC + a-GalCer + peptides ID were more likely to have a substantial melanoma specific CTL response than DC + peptides alone and 5 of the 11 patients demonstrated clinical anti-tumour activity. This is the first clinical study in melanoma patients that comprehensively demonstrates the adjuvant effects of NKT cell activation for enhancing tumour antigen specific CTL and to demonstrate clinical activity of a-GalCer + peptide pulsed DC in advanced melanoma.

Key Words: Adoptive therapy, Combination immunotherapy, Phase I.

Presenting author underlined. *Primary author in italics.*

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4-1BB AGONIST ANTIBODY TREATMENT INDUCES PRODUCTION OF IL-15 AND IL-27 BY ANTIGEN PRESENTING CELLS DRIVING THE FORMATION OF A NOVEL EOMES-DEPENDENT LINEAGE OF CYTOTOXIC CD4+ AND CD8+ T-CELLS

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Following treatment with 4-1BB agonist antibody, a novel population of KLRG1+ T-cells infiltrate the tumors of mice. Compared to their KLRG1- counterparts, these T-cells express high levels of cytotoxicity associated genes in both the CD4+ and CD8+ lineages. Further, these KLRG1+ cells demonstrate enhanced tumor-specific killing in vitro.

We have found that the phenotype of these KLRG1+ cells is dependent on high expression of the T-box transcription factor Eomesodermin (Eomes). Interestingly, only agonist antibodies targeting 4-1BB, not other TNFR family members can generate this phenotype in both CD4 and CD8 T-cells. The root of this difference appears to be that 4-1BB is expressed by antigen presenting cells (APC) which respond to its activation by producing cytokines which promote the development of these Eomes+KLRG1+ T-cells.

By analyzing changes in APC cytokine production in vivo using Taqman RNA analysis, as well as by using a series of gene knockout mice we have begun to identify the factors necessary to generate this novel T-cell lineage. Among these factors, IL-27 and IL-15 appear to be paramount; however, this analysis is not yet complete.

Understanding the nature of this novel lineage of highly tumoricidal T-cells in both tumor and pathogen-specific immunity may provide critical information for converting sub-optimal anti-tumor responses to therapeutically successful ones.

Key Words: CD4+ T cells, CTLA-4, Tumor infiltration lymphocytes.

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ACTIVATION OF THE IMMUNE SYSTEM BY SIPULEUCEL-T: PRELIMINARY DATA FROM THE OPENACT PHASE 2 TRIAL

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Background: Sipuleucel-T is an autologous cellular immunotherapy designed to stimulate an immune response in men with metastatic castrate resistant prostate cancer (mCRPC). Sipuleucel-T is made from peripheral blood mononuclear cells cultured ex vivo with a recombinant fusion protein, PA2024 (prostatic acid phosphatase [PAP] linked to granulocyte-macrophage colony-stimulating factor [GM-CSF]). In the pivotal Phase 3 IMPACT trial, sipuleucel-T demonstrated improved overall survival in men with asymptomatic or minimally symptomatic mCRPC. Immune response and safety data from OpenACT, an open label Phase 2 trial in 104 men with mCRPC, are presented.

Materials & Methods: Sipuleucel-T was administered every 2 weeks x3. Antigen presenting cell (APC) activation (CD54 upregulation) in the product was assessed by flow cytometry. In vivo responses to PA2024 and PAP antigens were assessed at baseline and 2 wks after the third infusion by IFNγ ELISPOT and 3H-thymidine T cell proliferation assays; humoral responses were measured by ELISA. Cytokines were profiled during manufacture and in subject serum before and after treatment (multiplex MSD assay).

Results: CD54 upregulation in sipuleucel-T products was greater at the second and third infusions relative to the first, suggesting a prime-boost phenomenon. Culture supernatants showed an increase in T cell activation-associated cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFNγ, and TNFα) after the first infusion. Cytokines associated with APCs (IL-8, IL-12p70, IL-1β, MCP-1, MIP-1β, TARC, and Eotaxin) were elevated. Compared to baseline, humoral responses against PAP and PA2024 after therapy were robust ($P<0.001$ for both). Post-treatment IFNγ ELISPOT responses to PA2024 and PAP were increased from baseline ($P<0.001$ and $P=0.073$, respectively) as were T cell proliferative responses ($P<0.001$ and $P=0.003$, respectively). Serum cytokines associated with immune activation increased from baseline (IL-6, TNFα, and IL-10 [$P<0.05$]). Prior docetaxel exposure (28% of treated subjects) did not adversely affect immune responses. Adverse events were comparable to those reported in the IMPACT trial.

Conclusions: Sipuleucel-T generates a prime-boost immune response in men with mCRPC by activating the immune system. The humoral response to PAP and newly reported serum cytokine profiles provide support for sipuleucel-T's proposed mechanism of action.

Key Words: Active immunotherapy, Cellular immunity, Prostate cancer.

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FORCED NF-κB IN T CELLS LEADS TO TUMOR REJECTION

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

NF-κB activity has been reported to be reduced in T cells from tumor-bearing hosts. Our previous results indicate that reduced NF-κB activation results in impaired survival of T cells, decreased Th1 and Th17 differentiation and increased iTreg differentiation. Mice with reduced T cell-NF-κB activity fail to reject cardiac and pancreatic islet allografts in the absence of any pharmacological treatment. We hypothesize that forced activation of NF-κB in T cells should have the opposite effect and promote T cell survival, facilitate Th1/Th17 differentiation and prevent iTreg differentiation, which would be beneficial to reject tumors.

We generated mice expressing a constitutively active form of IKKβ (CA-IKKβ) in T cells. Ectopic expression of CA-IKKβ resulted in phosphorylation of NF-κB. Transgene expression was limited to CD4+, CD8+ and NKT cells and T cells showed increased NF-κB activation and nuclear translocation. T cell numbers were comparable to littermate controls, but CA-IKKβ mice had fewer Tregs and increased frequency of activated T cells that produced IFNγ upon re-stimulation. When B16-SIY melanoma cells were injected subcutaneously, tumors grew progressively in control littermates, whereas they were rejected by mice expressing CA-IKKβ in T cells. CA-IKKβ expressing T cells were necessary for tumor control, as shown by antibody-mediated depletion of CD4+ and CD8+ T cells. Furthermore, adoptive transfer of CA-IKKβ-expressing, but not wild-type, T cells into immune-compromised (RAG-deficient) hosts prior to inoculation of tumor cells was sufficient for tumor control. Finally, enhanced tumor control was observed in immune-competent mice when fewer than 5% of T cells expressed CA-IKKβ.

Our results may potentially be translatable to the clinic and demonstrate NF-κB to be at the cross-roads of major T cell fate decisions that uniquely synergize for control of tumor growth.

Key Words: Tumor immunity, T cells.

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IL-13 MEDIATES INVASION AND METASTASIS THROUGH IL-13Rα2 VIA ERK/AP-1 PATHWAY IN VIVO MOUSE MODEL OF HUMAN OVARIAN CANCER

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Previously, we have demonstrated that a variety of human cancers such as glioblastoma multiforme, head and neck, prostate, breast, AIDS associated Kaposi's sarcoma and ovarian cancers overexpress IL-13Rα2, a high affinity receptor for IL-13, which can serve as a tumor antigen and a target for receptor directed anti-cancer therapy. To determine a function of IL-13Rα2, here we have examined invasion and metastasis of ovarian cancer through IL-13Rα2 in vitro and in vivo mouse models of human ovarian cancer in response to IL-13. We tested cell invasion and protease activity in IL-13Rα2-overexpressing and IL-13Rα2-negative ovarian tumor cell lines. We observed that IL-13 treatment significantly augmented both cell invasion and enzyme activities in only IL-13Rα2-positive cells but not in IL-13Rα2-negative cells in vitro. To delineate the mechanism of IL-13 induced invasion, we examined IL-13 induced signal transduction through ERK1/2 and AP-1 pathways and MMP activities in vitro. We found that IL-13 enhanced ERK1/2, AP-1 and MMP activities only in IL-13Rα2-positive cells but not in IL-13Rα2-negative cells. In contrast, other signaling pathways such as IRS1/2, PI3K and AKT were not activated and thus do not seem to play a critical role in IL-13 induced signaling in ovarian cancer cell lines. Highly specific inhibitors for MMP and AP-1 efficiently inhibited both invasion and protease activities without impacting the basal level invasion and protease activities in vitro. In an orthotopic mouse model of human ovarian cancer, IL-13Rα2-positive tumors metastasized to lymph nodes and peritoneum earlier than IL-13Rα2-negative tumors. IL-13Rα2-positive tumor bearing mice died earlier than mice with IL-13Rα2-negative tumor. In addition, intraperitoneal injection of IL-13 shortened the survival of IL-13Rα2-positive tumor bearing mice compared to IL-13Rα2-negative tumor bearing mice. IL-13Rα2-positive tumors and lymph node metastasis expressed higher levels of MMPs and higher ERK1/2 activation compared to IL-13Rα2-negative tumors. In conclusion, IL-13Rα2 is one of the key genes involved in ovarian cancer metastasis through activation of ERK/AP-1 pathway and that targeting IL-13Rα2 might not only directly kill primary tumors but also prevent cancer metastasis.

Key Words: Cytokine, Ovarian cancer.

Presenting author underlined. *Primary author in italics.*

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A PHASE I SAFETY AND IMMUNOGENICITY TRIAL OF MVA-BN®-HER2 VACCINE BASED IMMUNOTHERAPY IN HER-2-POSITIVE BREAST CANCER PATIENTS FOLLOWING ADJUVANT THERAPY

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Background: HER-2 targeted vaccine approaches show promise for the treatment of breast cancer. HER-2 is oncogenic, immunogenic, and over-expressed in 20-25% of human breast cancers. MVA-BN®-HER2 utilizes a poxviral vector that encodes a modified form of HER-2 (extracellular domain plus 2 tetanus toxoid peptide epitopes). The MVA-BN® vector is a highly attenuated vaccinia virus, non-replicating in humans, and an immunogenic vaccine. In previous trials in the metastatic setting, treatment with MVA-BN®-HER2 was able to induce a modest humoral and/or T-cell response in a majority of patients. The MVA-BN®-HER2 product in the current study utilizes a different promoter (Ps early/late), which augments early and total HER-2 antigen expression. Preclinical data in mice with the new construct demonstrated that immunization induced stronger immune responses and anti-tumor activity against HER-2 expressing tumors. Methods: The current trial is evaluating the safety and biological activity of MVA-BN®-HER2 in the adjuvant setting. Eligibility was determined by HER-2 over-expressing breast cancer (IHC 3+and/or FISH amplification >2+), without metastatic disease, and post adjuvant chemotherapy and traztuzumab treatment. Patients were vaccinated with a fixed dose of MVA-BN®-HER2 (1 x 108 TCID50) delivered by sc injection every 4 weeks x 6. Patients had peripheral blood leukapheresis pre-vaccine and post 3rd vaccination, and a phlebotomy after the 6th vaccination. Results: To date, 11/15 enrolled patients have completed 6 vaccinations. Vaccine was well tolerated with local injection site reactions as the main side effect. There was one possibly related SAE injection site reaction/cellulitis, but no patient discontinued due to an adverse event. No patient developed CHF, or significant decrease in cardiac ejection fraction per ECHO at 3 and 6 months. Immune evaluation by IFN-γ ELISPOT of samples from MVA-BN®-HER2-treated patients revealed that vaccine-induced T-cells were detectable in the majority of patients tested. HER-2 specific T-cells ranged from 20-120 spot forming cells (SFC) per 2×105 PBMC and tetanus toxin specific T-cells ranged from 12-80 SFC/2×105 PBMC. MVA-specific vaccine-induced T-cells were detected in all evaluated patients (15-550 SFC/2×105 PBMC). Conclusions: The data suggest that MVA-BN®-HER2 is well-tolerated, immunogenic, and support going forward with larger efficacy trials.

Key Words: Adjuvant, Breast cancer, Cancer virus.

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ATOMIC FORCE MICROSCOPE STUDY OF PACLITAXEL-INDUCED APOPTOSIS IN DAUDI CELLS

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Paclitaxel, one chemotherapeutic agent, can cause apoptosis in a variety of cancer cell lines. However little is known about the ultrastructure of cell surface from paclitaxel-induced apopotosis in Daudi cells. In this study, we investigated the apoptosis of paclitaxel on Daudi cells by atomic force microscope (AFM). The membrane ultrastructure of Daudi cell was analyzed by AFM. The growth inhibitory effect of Daudi cells was measured by trypan blue dye exclusion method and CCK8 assay. AnnexinV/PI double dyeing or PI dyeing was used to detect the cell apoptosis by Flow Cytometry. Trypan blue dye exclusion method and CCK8 assay showed that cell viability had a dose-dependent relationship with the paclitaxel concentration and time-dependent relationship. After being treated with paclitaxel, the size of cell membrane nanoparticles became small. The microstructure of the cell membrane displayed collapsing in the surface of the cells, the appearance of shrinkage and pores. Anexxin V/PI double dying assays indicated that the cell apoptotic rates in earlier period and advanced stage were obviously higher than control group. These results has great importance for visual diagnosis of early stage apoptosis in tumor cells in response to anti-cancer drugs, as well as studying interaction between drugs and cells.

Key Words: Apoptosis, Lymphoma.

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DAURICINE INDUCES APOPTOSIS AND INHIBITS PROLIFERATION IN LYMPHOMA CELLS

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Dauricine, a bioactive component of Asiatic Moonseed Rhizome, has been widely used to treat a large number of inflammatory diseases in traditional Chinese medicine. Dauricine is also known to exert anti-tumor effects, however, little is known about its effect on human lymphoma. In our study, we examined whether dauricine could inhibit the proliferation of lymphoma cells (Raji, Daudi) and induced apoptosis. The inhibition of cell growth was assessed by a modified MTT assay and counting cell number. Apoptosis was determined by morphological observation and Annexin V-FITC/ PI staining using flow cytometry. The mitochondrial membrane potential ($\Delta\Psi$m) was analyzed with the JC-1 and flow cytometry. Our results demonstrate that dauricine inhibited lymphoma cells proliferation and induced apoptosis in a dose- and time-dependent manner. In addition, Staining with the mitochondrial dye JC-1 revealed that dauricine induced a dose-dependent loss of $\Delta\Psi$m in the lymphoma cell lines. Taken together, our results demonstrate that dauricine inhibited lymphoma cells proliferation and induced cell apoptosis. These findings suggest

that dauricine is worthy of further in vivo exploration as a novel lymphoma chemopreventive and chemotherapeutic agent.

Key Words: Apoptosis, Lymphoma.

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THE ANTI-TUMOR EFFECTS OF A FOLATE-IMMUNOGLOBULIN CONJUGATE ARE ENHANCED BY CYTOKINE TREATMENT IN VITRO AND IN VIVO

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Folate conjugation is a means of selectively targeting therapeutics to folate receptor (FR) expressing cancer cells. A novel folate-bound immunoglobulin (F IgG) was tested for its ability to target natural killer (NK) cells to folate-receptor expressing cancer cells in the presence or absence of NK cell activating cytokines.

FR expression by the KB and HeLa cell lines was confirmed by Western Blot analysis and flow cytometry. Binding of F-IgG to NK cell Fc receptors led to increased phosphorylation of the epidermal growth factor related kinase (ERK) as measured by Western Blot. NK cell lysis of F-IgG-coated KB target cells was significantly enhanced following treatment of NK cells with IL-2, IL-12, IL-15, IL-18 and IL 21 (10ng/mL) ($p<0.005$). NK cell production of IFN-γ, RANTES and MIP-1α was also significantly enhanced by IL-12 in response to F IgG coated KB target cells as compared to control treated cells ($p<0.005$). Studies using an L1210JF murine leukemia model confirmed the anti-tumor activity of F-IgG and the ability of NK cell activating cytokines to significantly enhance its effects. NK cell depletion in tumor-bearing mice demonstrated that the anti-tumor effects of IL 12 and F IgG are dependent on NK cells and results in decreased tumor cell proliferation.

These studies indicate that F-IgG induces an immune response by NK cells against FR positive cancer cell lines and that cytokine treatment has a synergistic effect on this response both in vitro and in vivo. Thus, F-IgG has a potential to be utilized as a therapeutic antibody for the treatment of FR-positive cancers in combination with immune modulatory cytokines.

Key Words: Folate-IgG, IL-12, NK.

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DPX-SURVIVAC: AN ENHANCED PEPTIDE VACCINE TARGETING SURVIVIN FOR IMMUNOTHERAPY OF OVARIAN CANCER

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DPX-Survivac is a cancer vaccine that targets tumor-associated antigen survivin that promotes survival of tumor cells. Survivin is over-expressed in many types of cancers, and in particular, over 80% of ovarian cancers express this protein and that survivin expression seems to have prognostic value. DPX-Survivac cancer vaccine contains peptides derived from the protein sequence of survivin and these peptides have different HLA restrictions (HLA-A1, A2, A3, A24, B7) so that this combination of antigenic peptides covers approximately 85% of the North American population. Moreover, these peptide antigens have been selected for their superior immunogenicity across multiple HLA restrictions and formulated in the DepoVaxTM (DPX) platform that can increase the potency of peptide-based cancer vaccines. DPX contains both a T-helper peptide and proprietary poly nucleotide immune-stimulatory adjuvant. Previous clinical trials have separately shown the safety of these survivin-derived peptides in humans and our first-in-human trial using DPX-0907 vaccine (multi-peptide vaccine targeting breast, ovarian and prostate cancers) has proved that DPX platform is clinically safe. Enhanced immunogenicity to HLA-A2 survivin peptide was demonstrated using DPX-platform in immunized HLA-A*0201/H2Dd transgenic mice by measuring frequency of peptide-specific T cells. Our preclinical studies have also demonstrated safety and immunogenicity of multi-dose DPX-Survivac. Immunovaccine has obtained FDA approval for a phase 1/2 clinical trial of DPX-Survivac in ovarian cancer patients and the clinical site selection is in progress for study initiation in the fall of 2011. These studies use a combinatorial clinical trial design to limit immune suppression by regulatory T cells while enhancing vaccine-induced immunity. The clinical design involves a lead-in phase 1 trial evaluating the safety of DPX-Survivac, followed by a dose-finding safety/efficacy evaluation of two vaccine dose levels along with low dose cyclophosphamide (CPA). The study continues into a 2:1 randomized, placebo controlled phase 2 trial to assess the clinical benefit of low dose oral CPA in combination with DPX-Survivac in about 250 patients with advanced ovarian, fallopian tube and peritoneal cancer. CPA will be administered in a continual low dose (metronomic) as 'biologic responses modifier' that not only lowers the number of immune-suppressor cells but also is associated with minimum off-target effects. A broad immune-monitoring strategy will be followed to identify vaccine-induced antigen-specific immunity that can be correlated with clinical benefits.

Key Words: Active immunotherapy, Combination immunotherapy, Ovarian cancer.

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DEVELOPMENT OF A PERSONALIZED CELLULAR EX-VIVO CBL-B SILENCING CANCER IMMUNE THERAPY

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Despite significant recent progress, cancer immunotherapy efficacies are still limited, since tumors can down-modulate the immune system by generating immunosuppressive cytokine milieus, impeding T cell costimulation and inducing T regulatory cells.

The E3 ubiquitin ligase cbl-b has been identified as an important gatekeeper limiting T cell activation and costimulation and on the other hand mediates suppressive effects of TGF-beta on effector T cells. Concordantly, the immune system of cbl-b deficient mice can effectively fight tumors resulting in complete rejection, therefore validating cbl-b as an excellent target to enhance anti-tumor immune activity. We have recently shown in proof-of-concept experiments that cbl-b silenced human T cells mirror the phenotype of cbl-b deficient murine T cells, resulting in enhancement of cytokine production and proliferation.

To enable the clinical application of a cbl-b silencing treatment of human T cells to patients, we have developed a simple and reliable protocol that can be easily performed on any clinical unit that applies adoptive cell therapies to cancer patients. For this purpose, we have established a highly efficient transfection protocol using a commercial electroporation device enabling us to simultaneously transfect T, B, NK cells and monocytes with minimal cell damage. This protocol has several advantages, since it does not require any tedious cell purification steps employing magnetic selection but can be directly applied to freshly isolated PBMNCs as well as to in vitro cultured T cells. Moreover, by simultaneous silencing of all immune cells harboured in the PBMNCs, the situation moves closer to the immune compartment of genetically cbl-b deficient mice, where also specific increases of B and NKT cell activities have been demonstrated.

Additionally, we have screened for highly active siRNAs that can be produced in GMP quality. Using a proprietary siRNA design algorithm we obtained siRNAs that are able to shut down cbl-b expression for more than 7 days exceeding the silencing efficiency of commercial gold standard siRNAs that were used for the proof-of-concept experiments. Indeed, T cell activation further profits from this sustained silencing effect.

In summary, we have developed a reliable ex-vivo cbl-b silencing protocol in patients PBMNC that can be applied directly and easily to standard human leukocyte preparations and utilized for individual adoptive cell therapy alone or in conjunction with various active and passive approaches in cancer immunotherapy.

Key Words: Active immunotherapy, Adoptive therapy, Targeted therapeutics.

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A NOVEL BIOAVAILABLE SMALL MOLECULE CURCUMIN ANALOG FLLL100-P INHIBITS STAT3 SIGNALING AND INDUCES APOPTOSIS IN MELANOMA CELLS

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Constitutive activation of the Jak2/STAT3 pathway is critical for promoting an oncogenic and metastatic phenotype. We previously described FLLL32, a small molecule inhibitor that interacts with Jak2 and the STAT3 SH2 domain to inhibit its phosphorylation and dimerization. This compound was modeled based on the diketone form of curcumin. Although this lead compound induced caspase-dependent apoptosis in human melanoma cells at low micromolar concentrations, its poor solubility profile limited its dosing and efficacy in vivo. We hypothesized the FLLL32 lead compound could be structurally modified to provide greater solubility and bioavailability without compromising its STAT3-specificity and cytotoxicity. Molecular modeling and structure activity relationship studies were utilized to develop FLLL100-P, a phosphate pro-drug derivative of FLLL32. Upon in vivo administration of FLLL100-P to mice, the phosphate group is cleaved producing a biologically active compound, FLLL100. FLLL100-P is highly soluble, enabling concentrated dosing formulations in saline. Similar to FLLL32, in vitro studies confirm that the active FLLL100 compound inhibits in vitro JAK2 kinase activity, constitutive STAT3 phosphorylation (at Tyr705), and induces apoptosis of both V600E mutated and B-Raf wild type human melanoma cell lines at low micromolar doses. In contrast to curcumin, pre treatment of melanoma cells with FLLL32 or FLLL100 also did not inhibit IFN γ-induced pSTAT1. These data indicate FLLL100 showed greater specificity for STAT3 as compared to the homologous STAT1 protein and did not antagonize the action of clinically relevant cytokines. Pilot disposition studies in immune-competent ICR mice also suggest FLLL100 and FLLL32 have similar plasma pharmacokinetics at equivalent intravenous doses of 10mg/kg. Furthermore, no acute toxicity was observed following intravenous (IV) injection of FLLL100-P up to the maximum dose tested, 200mg/kg, indicating significantly higher doses can be achieved with the pro-drug. These data indicate that soluble and bioavailable small molecule STAT3 pathway inhibitors can be generated based on the molecular structure of curcumin. In vivo efficacy studies of these inhibitors in murine models of melanoma as single agents and in combination with immunotherapies are currently ongoing.

Key Words: Apoptosis, Melanoma, Targeted therapeutics.

Presenting author underlined. *Primary author in italics.*

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HIGH PD-1 LEVELS AT BASELINE ARE ASSOCIATED WITH UNFAVOURABLE CLINICAL OUTCOME IN A WILMS TUMOUR GENE 1 (WT1) PEPTIDE VACCINATION SETTING IN LEUKAEMIA PATIENTS

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Objectives: In the context of a phase I/II study to analyze the immunogenicity and toxicity of an HLA-A201-restricted WT1126-134 epitope vaccination in AML patients, we could previously demonstrate immunological and clinical responses but no correlation between tetramer or cytokine responses and outcome parameters was seen in the initial analyses. Therefore more detailed immunological analyses were performed in a subgroup of patients. We here report on the identification of the T cell exhaustion marker PD-1 as a potential limiting factor for vaccine efficacy.

Methods: After vaccination with 0.2mg WT1.126-134 peptide (day 3) admixed with 1 mg KLH, and 62.5µg GM-CSF (day 1-4) 16 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 PD-1 and CCR7 expression, induction of CD137, and the capacity to produce IFN-γ, TNFa and IL-2 and to mobilize CD107a, in response to the WT1 vaccine peptide were evaluated ex vivo. T cell response characteristics were correlated with progression-free survival (PFS).

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 or the activation marker CD137 and PFS. However, increased PD-1 values at baseline could be associated to influence PFS (p=0.05). Patients with PD-1 values exceeding 0.6% of CD3+CD8+ T cells (n=9; mean= 1.937%; median=0.88%; range 0.6% -6.01%) at baseline exhibited a median PFS of 132 days (range= 49 -201 days) while patients with low PD-1 frequencies (n=7, mean= 0.25%; median= 0.16%; range 0.058% -0.2%) exhibited a significant longer PFS of 149 days (range= 57 -1682(+) days). Another trend, underlining a possible reduced vaccine efficacy elicited by PD-1 was seen by comparison of differences in WT1-mRNA-levels at baseline and at week 10 in both groups. The low PD-1 subset exhibited a reduction of WT1-mRNA level (range = 0.016 - 1; mean = 0.514; median = 0.62), while the elevated PD-1 subset displayed an average increase in WT1-mRNA expression (range = 0.025 - 10; mean = 2.681; median = 0.95, p=0.05).

Conclusion: T-cell exhaustion at baseline, as characterized by high PD-1 expression, may limit the clinical efficacy of WT1-specific vaccination and underline the immunotherapeutic importance of protective blocking antibodies against PD-1 or PD-L1 in patients with leukemia.

Key Words: Cancer vaccine, Leukemia, PD-1.

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CLASSIFICATION OF MELANOMA CELL LINES ACCORDING TO IMMUNE MODULATORY PROPERTIES

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Objective: IFN-α responsiveness is hampered in PBMCs from melanoma patients. Although several factors may contribute, melanoma cells may directly be responsible. Thus, we tested 12 melanoma cell lines for their direct immune modulatory effects on PBMCs. Methods and results: PBMCs from 4 healthy donors were co-cultured for 7 days with melanoma cells in a trans-well system and phosphorylation of STAT1 (pSTAT1) before and after IFN-α stimulation was assessed in PBMCs subsets and melanoma lines. Baseline pSTAT1 was not changed significantly by co-culture. IFN-α-induced pSTAT1 in CD4 T cells was modulated differentially by the 12 melanoma cell lines. Five cell lines depressed IFN-α-induced pSTAT1 with high significance (p-value < 1x 104) as compared to PBMCs alone and were termed "Low", while the other 7 cell lines induced only mild down-regulation (p-value <0.01) and were termed "High". Similar finding were observed in CD8 T cells and monocytes. IFN-α-induced-pSTAT1 was significantly different in melanoma cells when comparing the low vs the high group (p-value<0.05). These results suggest that the low-group could modulate the IFN pathway response in PBMCs without direct contact while down-regulating their own response to IFN-α. Gene expression comparison between the two groups identified 6,136 differentially expressed transcripts (cutoff p-value < 0.05). Gene Ontology and Ingenuity Pathway Analysis suggested that the predominantly distinctive biological processes were response to immune stimulus and top pathways were G-protein coupled receptor signaling, primary immunodeficiency signaling and embryonic stem cell pluripotency, the latter up-regulated in the low-group including higher expression of OCT4 and UTF1. Conversely, most cancer growth pathways including RAS-BRAF-MTIF were active in the high-group. The low-group also down regulated antigen presentation and IFN signaling. None of canonical immune suppressive factors, such as TGFβ2, VEGF, PDGF was found significantly higher in the low-group. DNA copy number analysis demonstrated amplifications specific for the low-group in chromosome 12 and chromosome 8 for the high group (p-value <0.001). Conclusion: Melanomas are heterogeneous in modulating responsiveness of PBMCs to IFN-α. Melanoma cells with higher suppressive activity have higher proliferative properties and resemble undifferentiated stem cells. These differences are associated with distinct patterns of genetic imbalances whose significance is currently being investigated.

Key Words: Cellular immunity, Melanoma, Tumor microenvironment.

Presenting author underlined. *Priminary author in italics.*

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IMCMAGE1: A NOVEL BI-SPECIFIC BIOLOGIC TARGETING THE CANCER TESTIS ANTIGEN MAGE-A3

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Antigenic peptide fragments presented by HLA molecules on the surface of cancer cells constitute the largest class of cancer specific targets. CD8+ cytotoxic T cells of the immune system scan the HLA-peptide antigens being presented to them using their T Cell Receptor (TCR). Recognition and binding of the TCR to its target results in activation of the T cell and targeted killing of the antigen presenting cell. Unfortunately, unlike viral antigens, most cancer specific peptide antigens are identical to "self" and so T cells whose TCR recognise the antigen will have been deleted during thymic selection as a key defence against auto-immune disease. At the molecular level the consequence of thymic selection is that the cancer antigen specific TCRs that survive selection are typically much lower affinity than their viral specific brethren. This low affinity limits their ability to physically detect the antigen; a problem that is compounded by HLA down-regulation, an immune evasion strategy often employed by cancers.

In order to overcome the inherent limitations of the natural immune system and take advantage of the abundant, well validated, disease associated peptide antigens displayed on tumour cells we have developed a platform technology based on engineered TCRs. These bi-specific biologics, termed ImmTACs (Immune mobilising mTCR Against Cancer) combine soluble, affinity enhanced, TCRs specific for cancer associated antigenic peptide HLA complexes as a new class of targeting system with a potent anti-CD3 scFv based T cell redirection effector function.

Here we present a novel ImmTAC, IMCmage1, which has been designed to target the cancer testis antigen Mage A3, presented by HLA A1. Mage A3 is a member of the so called cancer testis family of antigens and is expressed on a wide range of cancer types including multiple myeloma, melanoma, NSCLC, prostate cancer, bladder cancer and oesophageal cancer amongst others. IMCmage1 has been shown to potently redirect T cell killing of a range of cancers and has been demonstrated to be cancer specific in a series of cross reactivity experiments against normal human primary cells. A Phase I clinical trial in multiple myeloma to assess tolerability and establish a Maximum Tolerated Dose is planned to commence in Q1 2012.

Key Words: CD8+ T cells, Phase I, Targeted therapeutics.

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COMBINED THERAPY WITH Vγ9Vδ2 T CELLS AND ZOLEDRONATE IN PATIENTS WITH ADVANCED SOLID TUMORS

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The majority of gamma/delta (γδ) T cells in human peripheral blood is of Vγ9Vδ2 phenotype and constitutes 1-5% of circulating lymphocytes. Immunotherapy with adoptively transferred, ex vivo expanded, autologous Vγ9Vδ2 T cells may be of therapeutic benefit in diverse malignancies due to their potent direct cytotoxicity toward tumor cells, synergistic cytotoxicity when combined with aminobisphosphonates, and enhancement of antibody dependent cell mediated cytotoxicity. In a pilot trail to examine the feasibility and safety of combined therapy, 18 patients with advanced solid tumors were treated with Zoledronate and ex vivo expanded, activated Vγ9Vδ2 T cells. In vivo trafficking of the adoptively transferred cells was studied by tracking Indium111-oxine labeled Vγ9Vδ2 T cells in a cohort of patients. Administered Vγ9Vδ2 T cells had an activated effector memory phenotype, expressed chemokine receptors predictive of homing to peripheral tissues and were cytotoxic in vitro against tumor targets. Adoptively transferred Vγ9Vδ2 T cells trafficked predominantly to lungs, liver and spleen and, in some patients, to metastatic tumor sites outside these organs. No dose-limiting toxicity was observed but most patients progressed on study therapy. However, three patients administered Vγ9Vδ2 T cells whilst continuing previously ineffective therapy had disease responses, suggesting an additive effect. The results demonstrate that therapy with aminobisphosphonate activated Vγ9Vδ2 T cells is feasible and well-tolerated but significant clinical benefits may result only when used in combination with other therapies.

Key Words: Adoptive therapy, Combination immunotherapy, Phase I.

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DENDRITIC CELLS TRANSFECTED WITH ERBB-2 RAT/HUMAN HYBRID PLASMID ELICIT AN EFFECTIVE T CELLS RESPONSE IN ERBB2+ CANCER PATIENTS

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Since ErbB-2 is overexpressed in many human cancers this molecule is an ideal candidate for developing cancer immunotherapy

protocols. Even if vaccination studies in animal models were successful in inhibiting tumor growth, in humans effective clinical results have not yet been obtained.

Here, we studied an alternative approach to overcome tolerance and elicit an efficient immune response against ErbB-2 in humans. We transfected dendritic cells (DCs) with DNA plasmids that express both human ErbB-2 and heterologous rat Neu extracellular and transmembrane sequences in separate plasmids or as single hybrid constructs that encode ErbB-2/Neu fusion proteins. In this way, Hu-DCs, R-DCs, HuRT-DCs, RHuT-DCs were generated from HLA-A2+ breast and pancreatic ErbB-2+ cancer patients and from HLA-A2+ age matched healthy donors and used to activate autologous T cells.

Whereas in healthy donors Hu-, HuRT- and RHuT-DCs were able to trigger IFN-γ release by autologous T cells, in cancer patients only RHuT- DCs elicited a significant IFN-γ response against immunodominant peptides. Interestingly, the induced T cells showed ability to in vitro kill ErbB-2+ tumor cells. Lower amount of IL-10 and TGFβ1 in the RHuT-DCs-T cells co-cultures could explain the effectiveness of RHuT plasmid to rescue specific T cells from tolerance in tumor patients.

These preliminary data suggest that a selective combination of rat and human ErbB-2 determinants could counteract the immunosuppressive status of tumor patients eliciting a broad spectrum of T cell potentially able to kill tumor cells.

Key Words: Cancer immunotherapy.

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CISH DEGRADATION OF HTRA1 REGULATES TGF-β SIGNALING

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T cell immunity to infectious and malignant diseases is intrinsically regulated by Suppressor of Cytokine Signaling (SOCS) members. Cytokine inducible SH2-containing protein (Cish), a SOCS member, is a purported Stat5 feedback inhibitor but its physiologic role and mechanism of action are poorly understood. We found that disruption of Cish dramatically enhanced CD8+ T cell cytokine production and in vivo tumor killing, but not Stat5 phosphorylation. Rather, we observed that Cish physically interacted with and degraded Htra1, a secreted protease of TGF-β family members. Cish promoted TGF-β/SMAD signaling and Htra1 destruction was critical for this regulation. Furthermore, recombinant Htra1 blocked TGF-β signaling and enhanced CD8+ T cell immunity in the presence of Cish. TGF-β antagonizes Stat5 target gene expression and the elucidation of the Cish/Htra1 nexus reveals how these signals are integrated to control cellular immunity. These findings may have implications in the treatment of infectious or malignant diseases.

Key Words: CD8+ T cells, Cellular immunity, Cytokine.

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CANCER TESTIS ANTIGENS AS PROGNOSTIC BIOMARKERS FOR BREAST CANCER PATIENTS

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We have previously reported that the presence of a distinct immune function gene signature network at the tumor lesions of patients with early stage breast cancer could predict relapse-free survival following conventional therapies. We hypothesized that expression of cancer testis antigens (CTA) may be responsible for converting weakly immunogenic breast tumors into highly immunogenic tumors, and result in relapse-free survival. To test this hypothesis, we performed qRT-PCR analysis of RNA extracted from tumor lesions of patients with breast cancer from which we compared CTA expression levels of those who relapsed within 1-3 years with those who remained relapse-free during 5-7 years follow-up. We detected an increased expression of a number of CTA in tumor lesions of patients who remained relapse-free but not in those with tumor relapse. We also showed that treatment of human breast tumor cell lines with a demethylating agent, Decitabine, induced expression of CTA in the tumors. Altogether, these data suggest that lack of CTA expression in tumor lesions of breast cancer patients at the time of diagnosis may predict high risk of tumor relapse, and that using Decitabine in a neoadjuvant setting may convert patients with high risk into those with low risk of tumor relapse.

Key Words: Breast cancer, Cancer immunotherapy, relapse.

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PHASE I CLINICAL TRIALS IN CANCER VACCINE DEVELOPMENT DO NOT DETERMINE DOSE NEITHER BASED ON SAFETY NOR ON BIOLOGICAL ACTIVITY

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Phase I clinical trials are generally conducted to identify the MTD and the optimal efficacious dose in a traditional dose escalation design. However, this design may not apply to certain therapies such as cancer vaccines, given their unique mechanism of action and the profile of their clinical outcome. Based on these factors FDA is in the process of establishing the guidelines for therapeutic cancer vaccines development. Nevertheless, the question of whether the conventional design could still be used is a challenge. To address this question we analyzed the toxicity profile in 241 therapeutic cancer vaccine phase 1, phase1/2, and pilot clinical trials conducted between 1990 and 2011. In trials that used dose escalation design we addressed the relationship between vaccine dose and toxicity and the ability of dose escalation to determine biologically active dose (BAD). Amongst 241 trials 62 grade 3/4 vaccine related systemic toxicities were reported in 4952 treated patients (1.25% toxicity rate). The number of grade 3/4 toxicities was also analyzed

in relation to the number of the administered vaccines in 206 trials out of the 241 trials. Based on this analysis, 4,024 patients received 21,835 vaccines and experienced 43 grade 3/4 systemic vaccine related toxicities (0.2% toxicity rate). In order to study the dose-toxicity relationship, we analyzed all trials that used dose escalation design (127/241 trials). Twenty-two of 127 dose escalating trials reported 40 grade 3/4 systemic vaccine related toxicities with only 10 toxicities occurred at the highest dose level. Interestingly, only 3 trials out of 127 dose escalating trials reported DLT. One out of 17 allogeneic vaccine trials reported a DLT related to the adjuvant, and two out of 37 bacterial vectors vaccine trials reported DLTs related to the vaccines. Furthermore, we analyzed the dose-immune response relationship in 106 trials that included immune response as a secondary endpoint out of the 127 dose escalating trials. We also included 10 additional trials designed to determine BAD by immune response as a primary endpoint. Out of 116 trials, only 2 trials showed a statistically significant dose immune response correlation (a peptide vaccine and an anti-idiotypic vaccine). Our analysis suggests that potential serious toxicity in vaccines therapy is extremely low and the toxicity or biologic activity do not correlate with dose levels based on the traditional dose escalation design. Accordingly, conventional dose escalation phase I design is not suitable for cancer vaccine studies with few exceptions. Alternative designs to determine vaccine dose should be developed. We will explore alternative designs to address BAD based on immunologic activity.

Key Words: Phase I, Cancer Vaccine, Toxicity.

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INFLUENCE OF MELANOMA HOST CELL FACTORS ON REPLICATION AND EARLY GENE EXPRESSION OF ONCOLYTIC VACCINIA VIRUS ISOLATES

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Vaccinia virus (VACV) has the ability to take up great amounts of foreign DNA in its genome without major impact on its infection and replication capabilities. A combination of VACVs natural tumor tropism and oncolytic features with insertion of therapeutic transgenes could serve as a promising tool in cancer treatment. VACV replication efficiency plays also a crucial role for the therapeutic outcome.

The aim of the current study was to find and analyze correlations between viral replication, viral gene expression and the respective host response at an early time point after infection.

VACV replication in responding and non-responding melanoma cells was determined by plaque assay analysis. Viral gene expression in infected cells was evaluated using customized VACV expression arrays whereas host gene expression was analyzed via human whole genome (36 K) array platforms.

Two melanoma cell lines derived from the same patient showed a time point specific gene expression pattern not only in VACV infected samples, but also in uninfected controls. Designed sequential statistical approaches characterized 703 human genes which change specifically due to virus infection. Different VACV isolates showed altering viral gene expression levels as early as 2 hours post infection (hpi). The hierarchy of levels of gene expression in a set of 10 viral early genes matched perfectly with the respective replication efficiency at 2 hpi. Moreover, correlation analyses between host gene expression and the viral candidate genes revealed a strong negative or positive correlation with a set of human genes.

The results indicate a direct correlation between viral replication, early gene expression and the respective host response. The characterization of human target genes that influence viral replication could help to develop novel recombinant vaccinia viruses with improved features to enhance replication rate and hence also trigger the therapeutic outcome.

Key Words: Melanoma.

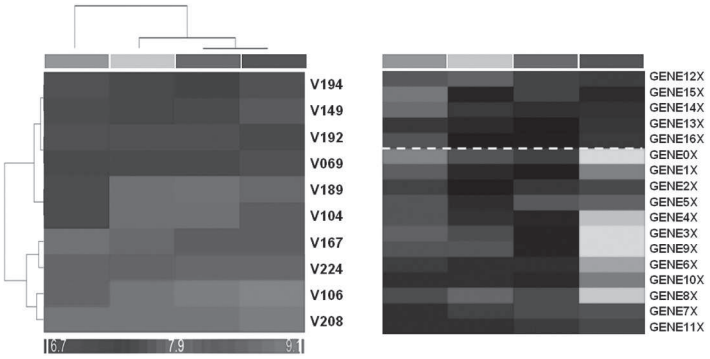

Figure 1. *Correlation between early VACV and human gene expression. Viral gene expression of 10 candidate genes at 2 hours post infection (left) correlated with the respective replication efficiency of the 4 VACV isolates analyzed. Early human gene expression (right) was correlated with the viral candidate genes (Pearson Correlation, R2=0.7 for ≥ 6 out of 10 VV genes).*

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CALORIC RESTRICTION RESPONSES CAN MAINTAIN OX40-MEDIATED IMMUNE RESPONSES DURING AGING

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Age-related changes accumulate in both the innate and adaptive arms of immunity to generate a state of immune deficiency that can influence immunotherapy. This deficiency was evident as we have observed a dramatic decrease in tumor immunity in mice 12- or 20-months old compared to younger (two- and six-month old)

mice after treatment with the immunotherapeutic anti-OX40. In addition, we observed age-related deficiencies in the T cell priming environment, as adoptively transferred young transgenic TCR T cells (DO11.10) exhibited a decrease in expansion when primed with antigen and anti-OX40 in older hosts. Thus, approaches to restore and/or maintain immunity during aging are needed to boost the efficacy of immunotherapies in older patients. We hypothesize that the induction of a caloric restriction response, mediated by traditional caloric restriction protocols (CR) or caloric restriction mimetics, will restore and/or maintain OX40-mediated immune responses during aging. Two caloric restriction approaches were used. In the first approach, 12-month old mice were subjected to CR (40% decrease in calories, but not essential nutrients) for two months to test if immunity can be restored in older mice. In a second approach, CR was intiated in mice at two months of age and continued until they reached 12 months of age, to determine if immunity can be maintained during aging. Finally, six month old mice had the compound resveratrol, a natural chemical that mimics the biological effects of CR without reducing caloric intake, introduced into their diets and continued until mice reached 12 month of age to test if this CR mimetic could maintain immunity. These CR or resveratrol fed mice were either 1) adoptively transferred with DO11.10 T cells (two-month old) and primed with antigen and anti-OX40 or 2) challenged with tumor (MCA205 or EMT6) followed by treatment with anti-OX40. Our results demonstrate a caloric restriction response administered at a young age can maintain anti-OX40-mediated tumor immunity at older ages and short-term caloric restriction responses fail to restore T cell immune responses in older mice. Thus, long-term restriction of calories may maintain immunological fitness and increase the effectiveness of immune-based cancer therapies in aging patients.

Key Words: Animal model, CD4+ T cells, Cellular immunity.

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IDENTIFICATION OF PREDICTIVE BIOMARKERS IN CANCER PATIENTS RECEIVING PERSONALIZED PEPTIDE VACCINATION

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We have previously demonstrated the feasibility of our novel immunotherapeutic approach, personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered on the basis of the pre-existing host immunity before vaccination. However, it is critical to identify reliable biomarkers for selecting patients who would most likely benefit from PPV. since only a subset of patients show clinical responses to this treatment. In order to further improve the clinical efficacy of PPV, we tried to identify pre-vaccination biomarkers for predicting prognosis in advanced cancer patients receiving PPV. In castration-resistant prostate cancer, a comprehensive study of soluble factors and gene expression profiles in peripheral blood demonstrated that higher IL-6 level and granulocyte signature, which may be attributed to granulocytic myeloid-derived suppressor cells (MDSCs), were closely associated with poor prognosis in the vaccinated patients (n = 40). In biliary tract cancer, multivariate Cox regression analysis showed that higher IL-6 and lower albumin levels before vaccination and less numbers of peptides selected for vaccination were significantly unfavorable factors for overall survival (OS) in the vaccinated patients (n =25). In non-small cell lung cancer patients receiving PPV (n = 41), higher level of another inflammatory factor, CRP, was significantly unfavorable for OS. Taken together, these findings suggested that less inflammation, better nutrition, and pre-existing immune responses to greater numbers of antigens before vaccination may contribute to better responses to PPV. Evaluation of inflammatory and nutritional status and pre-existing host immunity to antigens before vaccination could thus be useful for selecting cancer patients, who would benefit from PPV. Our findings might provide a new insight for designing and improving cancer vaccines. The detailed information and future perspectives will be presented.

Key Words: Cancer vaccine, Cytokine, Myeloid derived suppressor cell.

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VACCINATION WITH THE HER2-DERIVED E75 PEPTIDE VACCINE IN BREAST CANCER PATIENTS MAY CONFER GREATER BENEFIT TO PATIENTS WITH LESS AGGRESSIVE DISEASE

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Background: We have conducted phase II trials of the HER2-derived E75 peptide vaccine given to breast cancer patients in the adjuvant setting. E75 (HER2:369-377) is an immunogenic HLA-A2/A3-restricted peptide from the extracellular domain of the HER2 protein. When combined with GM-CSF (immunoadjuvant) as a vaccine, E75 may reduce breast cancer recurrences. This analysis evaluates clinical response in patients with varying risk determined using Adjuvant! Online.

Methods: Node positive or high-risk node negative breast cancer patients with any level of HER2 expression (IHC 1+, 2+, or 3+), rendered disease-free after standard adjuvant therapies were enrolled. HLA-A2/A3+ patients were vaccinated with E75+GM-CSF while HLA-A2/A3- patients served as controls. The vaccine was given as 4-6 monthly intradermal inoculations. The primary endpoint of the trial was disease-free survival. Clinicopathologic factors for each enrolled patient were used to calculate the Adjuvant! Online 10 year mortality score (Adjuvant! for Breast Cancer, Version 8.0). p-values were calculated using the log-rank and chi-square tests.

Results: 187 patients have been enrolled (vaccine=108, control=79). With 60 months median follow-up, the vaccine group experienced a 10.6% recurrence rate compared to 20.3% in the control group (48% risk reduction, p=0.098). Recurrence rates for vaccine and control patients with different disease features (nodal status, HER2 expression, tumor grade, and hormone receptor status) are shown (see table). Vaccinated patients had a mean Adjuvant! Online mortality score of 23.9 (SE±2.1) compared to 28.0 (SE±2.9) for control patients (p=0.26). Vaccine patients with mortality scores ≤55 had a 6.2% recurrence rate (6/91) compared to a 15.6% (10/64) in controls (p=0.05)

Conclusions: In a phase II trial evaluating the HER2-derived E75 peptide vaccine in breast cancer patients in the adjuvant setting, vaccinated patients with node negative disease, lower levels of HER2 expression, lower grade tumors, hormone receptor positivity, or lower Adjuvant! Online scores appear to have lower rates of breast cancer recurrence. This finding suggests that patients with less aggressive disease features may derive greater clinical benefit from vaccination.

Key Words: Breast cancer, Cancer vaccine, Phase II.

Table 1. *Recurrence rates for different disease features*

		Control		Vaccine			
	Disease Feature	n	Recurrence Rate (%)	n	Recurrence Rate (%)	Risk Reduction (%)	p (log rank)
All Patients		79	20.3	108	10.6	48	0.098
Less Aggressive	Node Negative	35	18.4	55	5.5	70	0.19
	HER2 Low Expression	52	25.1	69	10.6	58	0.06
	Low or Intermediate Grade	46	21.2	63	4.8	77	0.01
	ER or PR positive	65	19.7	74	8.6	56	0.07
More Aggressive	Node Positive	53	22.9	44	15.8	31	0.31
	HER2 Over-expression	19	15.8	32	12.6	20	0.68
	High Grade	20	20.8	42	20.6	1	0.96
	ER and PR negative	14	25.0	33	15.2	39	0.65

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COMPARISON OF TKI MEDIATED EFFECTS ON THE MECHANISM OF T CELL ACTIVATION

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Tyrosine kinase inhibitors (TKI) have been successfully implemented as front-line therapy for the treatment of renal cell carcinoma (RCC) and provide significant objective clinical responses and a longer progression-free survival of patients. Different TKIs are able to modulate immune responses by modulating the frequency and function of T cell subpopulations, dendritic cells (DC) as well as myeloid-derived suppressor cells (MDSC). Thus, for the optimised clinical use of these inhibitors a better understanding of the impact of TKIs on specific anti-tumor responses as well as on the general immune activity is required. Despite immunmodulatory effects of sunitinib and sorafenib were studied, little information is available about the immune-adjuvant activities of axitinib. Thus, the aim of this study was to determine the effects of axitinib and sunitinib on the cell growth, apoptosis, cell cycle arrest and activation of T cells using the immortalized Jurkat T cell line and of peripheral blood mononuclear cells (PBMC). Axitinib as well as sunitinib inhibited proliferation as well as metabolic activity of Jurkat cells in a dose-dependent manner. The axitinib-mediated growth inhibition was biphasic with IC50-values of 0.20 ± 0.05 µM for the first phase and 21.7 ± 2.4 µM for the second phase after 48 h and 72 h axitinib treatment, whereas no effect of axitinib on the viability of stimulated PBMC could be detected. In contrast, the sunitinib-mediated growth inhibition was shown as single phase with IC50-values of 3.7 ± 0.5 µM for Jurkat cells and 5.4 ± 0.5 µM for PBMC. Cell cycle arrest in G2/M phase was only detected in the presence of axitinib, but not for sunitinib. Axitinib-induced effects revealed a slight up-regulation of CD69 during the activation of Jurkat cells, whereas sunitinib treatment leads to a strong down-regulation of CD69. Furthermore, axitinib- and sunitinib-induced alterations of the protein expression profile of activated and unstimulated Jurkat cells was determined using a 2D-based proteomic approach. So far > 20 differentially expressed protein spots were detected in (un) stimulated Jurkat cells upon axitinib and sunitinib treatment. Based on our results both TKIs differentially afect the function of T cells. Ongoing analysis of axitinib- and sunitinib-regulated proteins will shed light into the mechanisms of action of these TKIs in T cells.

Key Words: Antiangiogenesis, CD8+ T cells, Targeted therapeutics.

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DEFICIENCY OF CD47 IN THE TUMOR MICROENVIRONMENT ENHANCES TUMOR RESPONSES TO IONIZING RADIATION BY THE ACTIVATION OF PROTECTIVE AUTOPHAGY IN CYTOTOXIC MACROPHAGES

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Over half of all cancer patients will receive radiation therapy as part of their treatment regimen, however achieving a curative response is limited by off-target toxicities in normal cells. Our laboratory has reported that blockade of the thrombospondin-1 and its receptor CD47 results in the radioprotection of normal tissue including muscle tissue, endothelial cells and hematopoetic progenitors. Moreover, suppression of CD47 in combination with ionizing radiation (IR) enhances the radiation-induced delay in a syngeneic melanoma mouse model. We now report that irradiated B16 melanoma tumors grown in a CD47-/- mice show a significant reduction in tumor volume when compared to tumors in irradiated WT mice (369.7±123.3 WT+IR vs 185.7±49.47mm3). These data indicates that a CD47-/- tumor microenvironment enhances radiation-induced tumor growth delay. Clonogenic assays demonstrate that blockade of CD47 protects circulating hematopoietic progenitors

including increase in survival of macrophage progenitors by over 65%. Blockade of CD47 in irradiated cultured B16 melanoma cells resulted in enhanced mouse RAW macrophage mediated cancer cell cytotoxicity when compared to irradiated and unirradiated counterparts. This effect also extends to differentiated U937 macrophage killing of MDA-MB-231 human breast cancer cells. Therefore, blockade of CD47 protects cytotoxic macrophages from IR and enhances tumor cytotoxicity to reduce tumor growth. Further studies indicate that the observed protection of macrophages is mediated by an increase in the expression of genes involved in the activation of autophagy. Autophagy is metabolic processes were the cell catabolizes its own components and relocates nutrients to essential processes normally ensuring cell survival. Blockade of CD47 increased the expression of beclin-1, autophagy related gene-5 (ATG5) and microtubule associated protein light chain-3 (LC3) in cultured macrophages after IR. Therefore blockade of CD47 may increase the survival of tumor associated macrophages after IR, which in turn would be cytotoxic to cancer cells and result in reduced tumor growth. These findings indicate that agents targeting TSP1/CD47 may allow for more aggressive application of IR in the treatment of cancer and increase the percentage of curative responses.

Key Words: Chemotherapy, Macrophages, Tumor microenvironment.

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TRAIL IS STERICALLY INCAPABLE OF ENGAGING DEATH RECEPTORS IN AN AUTOCRINE FASHION: IMPLICATIONS FOR TRAIL-BASED CANCER IMMUNOTHERAPIES

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Background: TRAIL is a member of the TNF superfamily and has been shown to be critically important as a key regulator of lymphocyte homeostasis and as tumor surveillance cytokine. Its biological activity is mediated in the fluid phase and by a membrane associated mechanism. Membrane bound wild type (wt) TRAIL might be expressed along with its receptors (e.g., DR5) on immune effector cells and on engineered mesenchymal stem cells (MSCs) used for immunotherapies. This raises an immediate concern: How can a cell survive this putatively deadly ligand/receptor interaction?

Methods: We recently established a genetically fused TRAIL trimer (TR3) as a powerful cancer therapeutic. We since generated cancer specific TR3 forms targeted to the tumor marker mesothelin via single chain antibodies (scFv), with and without a spacer separating the targeting from the effector domain of the fusion proteins. Using these constructs, we identified a previously unrecognized mechanism by which cells could evade apoptosis even when TRAIL and its receptors are concomitantly expressed. Since mesothelin targeted TR3 essentially mimics a membrane anchored form of TRAIL, we genetically engineered membrane anchored TR3 variants and followed their expression in HEK293T cells by flow cytometry and compared these patterns to that of wt TRAIL.

Results: The two mesothelin targeted TR3 forms killed their targets much more efficiently but to the same degree. However, we did notice substantial phenotypic differences in the way the two TR3 constructs killed their targets. In the absence of a spacer, an unexpected 7-fold increase in mesothelin positive target cells was observed whereas the spacer containing therapeutic showed a 7-fold decrease in mesothelin expressing cells. When DR5 positive 293T cells were transfected with wt TRAIL and membrane anchored TR3 variants (+ or - spacer) and stained for TRAIL, the cells expressing spacer deficient (and wt TRAIL) gave a similar staining pattern, whereas the spacer-containing cells showed a lack of TRAIL signal where DR5 was normally detected.

Conclusions: Our data show that wt TRAIL and spacer-deficient membrane bound TR3 are incapable of engaging their own receptors due to steric constraints and therefore only allow killing via a bystander mechanism. Conversely, artificially lifting TR3 to a more distal membrane position enables such an interaction and allows bystander and self-killing. In addition to contributing to our understanding basic TRAIL biology, these findings have implications for the design and choice of more effective cancer immunotherapies based on membrane anchored forms of TR3.

Key Words: Apoptosis, Targeted therapeutics.

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TARGETING THE TUMOR-DRAINING LYMPH NODE WITH ADJUVANT-LOADED NANOPARTICLES FOR CANCER IMMUNOTHERAPY

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The tumor-draining lymph node (TDLN) is thought to participate in cancer progression by enabling metastatic dissemination. However, accumulating evidence implicates the TDLN in tumor-induced immune escape, suggesting it might represent a novel target for drug delivery schemes aiming to reverse tumor immune tolerance. We hypothesized that we might alter tumor fate by targeted delivery of adjuvants (Adjs) to the TDLN in order to reshape immune responses against the tumor and impair tumor progression. To accomplish this, we implemented poly(propylene sulfide)-core, Pluronic-corona nanoparticles (NPs) recently developed by our laboratory, that target immune cells within the lymph node (LN) after intradermal administration. Adjs paclitaxel and CpG were either incorporated into the NP core by solvent dispersion or conjugated to the Pluronic corona, respectively. Adjuvant-bearing NPs (Adj-NPs) were found to retain the immune stimulatory capacity of the free adjuvant in vitro and induce inflammation and infiltration into ipsilateral (draining), but not contralateral (non-draining), LNs 24 h after intradermal injection in C57Bl6 mice. When applied to the TDLN via intradermal administration in the arm ipsilateral to a B16F10 melanoma tumor,

Adj-NP treatment reduced tumor growth and reshaped the immune milieu inside both the TDLN and the tumor itself, including increasing the frequency of mature CD11c+ cells in the TDLN, increasing the frequency of Th1-biased CXCR3+ cells in the TDLN, increasing the frequency of activated and antigen-specific CD8+ T cells in the tumor, and decreasing the frequency of regulatory T cells in both the TDLNs and tumor. Furthermore, reduced tumor growth resulted from the targeted delivery of Adj to the TDLN rather than systemic immune activation as application of Adj-NPs to the contralateral (non-tumor draining) LNs failed to alter tumor growth. Together, these data suggest that the suppressive immune environment within the TDLN plays a pivotal role immune response against a tumor as well as implicate the TDLN as a novel drug delivery target for immunotherapy of solid tumors.

Key Words: Adjuvant, Combination immunotherapy, Targeted therapeutics.

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IRF5 GENE POLYMORPHISM IN MELANOMA

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Background. Prediction of patient with melanoma responsiveness to immunotherapy is uncertain. A connection between autoimmunity and benefit of interferon alpha-2b therapy was recently suggested. IRF5 is a transcription factor involved in the type I interferon and the toll-like receptor signaling. Previously, IRF5 has been found associated with several autoimmune diseases and, therefore, is a good candidate for autoimmunity. Thus, we examined whether polymorphisms of IRF5 associated with autoimmunity might also be associated with immune responsiveness of melanoma.

Method. Four single nucleotide polymorphisms (SNPs) and one insertion-deletion in the IRF5 gene were genotyped by sequencing in 142 Tumor Infiltrating Lymphocytes (TILs) from melanoma patients. Haploview v3.2 was used to generate haplotype frequencies and calculate the significance of association. Gene-expression profiling was assessed by Affymetrix Human Gene ST 1.0 array.

Results. The genotype and allelic frequency distribution showed significant differences between responders vs. non-responders. Presence of GG genotype in rs10954213 and rs11770589 was higher in non-responders (28%-10%, P=0.0076; 42%-18%, P=0.0051). Presence of exon 6 deletion was higher in responders (27%) than in non-responders patients (17%, P=0.0076). The allele rs10954213 A and rs11770589 A frequencies are higher in responders (.069 vs 0.37, P=0.000007, OR=3.08, 95%CIs =1.86-5.103 and 0.54 to 0.37, P=0.003, OR=1.99, 95%CIs=1.24-3.19). Exon 6 insertion was higher in non-responders patients (63% vs. 46%, P=0.003). The presence of rs10954213 and rs11770589 G allele and exon 6 insertion was associated to non-response (p=0.0046, p=0.0016 and p=0.0016 respectively). Exon 6 indel is in linkage disequilibrium with all the other IRF5 SNPs beside rs2004640 in both responders and non-responders groups. Haplotype analysis defined as negative prognostic factor rs10954213 G, rs11770589 G, rs6953165 G, rs2004640 T, exon6 insertion (p=0.009) in agreement with resistance to development of autoimmunity. mRNA variation by gene expression analysis on the same TILs did not correlate with IRF5 polymorphisms and was not associated with response to therapy.

Conclusions. This study is the first to analyze associations between melanoma immune responsiveness and IRF5 polymorphisms. The results support a correlation between IFN-mediated autoimmunity and melanoma immune responsiveness.

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

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COMPREHENSIVE CHARACTERIZATION OF HLA-A2 ASSOCIATED PEPTIDES EXPRESSED BY GLIOBLASTOMA IN VIVO, AND THEIR RECOGNITION BY CYTOTOXIC T CELLS FROM GLIOBLASTOMA PATIENTS

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T-cell mediated cancer immunotherapy will ideally target multiple tumor associated antigens to avoid the possibility of immune escape. To define target antigens expressed by glioblastoma cells in vivo, we used a novel combination of cutting-edge technologies to elute and sequence HLA-bound peptides from surgically resected tumors. We identified over 3000 HLA-A2 restricted peptides, from which we selected 10 peptides using criteria we consider to be critical for successful glioblastoma targeting. Specifically, the antigens from which these peptides were derived were implicated in tumorigenesis, they were overexpressed in glioblastoma compared with normal brain and other tissues, and all peptides were immunogenic in vitro when T cells from healthy HLA-$A2^+$ donors were tested. We further validated the relevance of the chosen peptides for immunotherapy by demonstrating specific reactivity to all of the peptides by T cells from patients with glioblastoma, and by generating cytotoxic $CD8^+$ T cell clones that efficiently killed antigen-expressing tumor cells. Moreover, $CD8^+$ T cells specific for a brevican epitope were isolated from a tumor biopsy in one patient, showing that, for this antigen, spontaneously elicited specific T cells had infiltrated the brain and had been retained in the tumor bed. Our comprehensive ex vivo and in vitro assessment of glioblastoma antigens have enabled us to design a multipeptide glioblastoma vaccine, IMA950, for clinical evaluation, and will offer future opportunities for further rationally targeted immunotherapies.

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

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IMCgp100: A NOVEL BI-SPECIFIC BIOLOGIC FOR THE TREATMENT OF MALIGNANT MELANOMA

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IMCgp100 is a novel bi-specific biologic comprising a soluble, affinity enhanced, T cell receptor (TCR) specific for the melanoma associated antigen gp100, fused to an anti-CD3 specific antibody fragment (scFv). IMCgp100 is being developed for the treatment of malignant melanoma. The engineered TCR portion of the drug targets the gp100 antigen, which is overexpressed and presented by HLA A2 on the surface of melanoma cells thereby effectively coating the melanoma cells in CD3 specific antibody fragments. The anti-CD3 scFv portion captures and redirects any T cells in physical contact with the melanoma to kill it. IMCgp100 has been demonstrated to potently redirect T cells within late stage cancer patient blood against significantly HLA down-regulated melanoma cells, even in the presence of high numbers of regulatory T cells.

This novel immunotherapeutic is currently under investigation as part of a Phase 1, open label, dose-finding study to assess the safety and tolerability in patients with unresectable Stage III/ Stage IV malignant melanoma. Patients will be HLA A2 positive, have an ECOG performance status of ≤1 and must not have high volume disease, brain metastases, a history of adult seizures, uveitis or a known immunosuppressive condition. The study will be conducted in 2 parts; a dose-escalation phase (Part 1) and a dose-expansion phase (Part 2). During the dose-escalation phase of the study, cohorts of 3 patients will receive a single intravenous infusion of IMCgp100 over a four hour period. Safety, tolerability, pharmacokinetic, pharmacodynamic, and clinical activity will be assessed and the maximum tolerated dose (MTD) established. Subsequently, Part 2 will enroll up to 20 patients using the dose established in Part 1 to further evaluate safety, tolerability, PK, PD, and to identify evidence of clinical efficacy. In the dose-expansion part of the study patients will receive an intravenous infusion of IMCgp100 over a four hour period on days 1, 8, 15, 22, 29 and 36 of a 66 day cycle. The study is currently actively enrolling.

Key Words: CD8+ T cells, Melanoma, Phase I.

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THE COMBINATION OF DASATINIB AND ANTI-OX40 ANTIBODY ENHANCES THE ANTI-TUMOR RESPONSE IN THE P815 MASTOCYTOMA TUMOR MODEL BY INCREASING TUMOR ANTIGEN-SPECIFIC EFFECTOR CELLS INFILTRATION INTO THE TUMOR

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Targeted therapy and immunotherapy are two of the most promising therapies for cancers. Existing targeted therapy drugs can induce transient regressions of large tumors but rarely achieve complete and durable responses. Immunotherapy can induce durable anti-tumor immune responses but response rates are low. Since these two therapies have complementary strengths and weaknesses, we hypothesized that the combination of these two therapies could improve therapeutic efficacy for cancers. In this study, we utilized the P815 mastocytoma tumor model to explore the possibility of combining them. Dasatinib is a small-molecule tyrosine kinase inhibitor which can strongly inhibit the activity of the mutant c-kit receptor in P815. We observed that 3 days of dasatinib treatment delayed the growth of P815 tumors but only improved the survival of the mice slightly. The combination of dasatinib and anti-OX40 antibody resulted in much better therapeutic efficacy than either drug alone. Most of the mice were cured. This therapeutic effect was T cell-dependent, since depletion of CD4 or CD8 positive T cells significantly compromised the therapeutic effect. Using P1A tetramer staining and IFN-γ intracellular staining, we found that dasatinib significantly enhanced tumor antigen-specific T cell responses. That may be caused by decreased level of Treg cells after dasatinib treatment. By isolating and analyzing the tumor infiltrating lymphocytes, we found that this combined regimen provided synergistic effects on the accumulation of tumor antigen-specific T cells in the tumor microenvironment. Realtime PCR showed that the combined regimen significantly up-regulated expression of CXCL9, 10 and 11 which are IFN-γ-induced Th1 chemokines. Since anti-OX40 has been shown to induce the expression of some Th1 chemokines and increase the infiltration of T effector cells in the tumor microenvironment, we postulate in our tumor model that: 1)dasatinib treatment increased the tumor antigen-specific T cell responses, and 2)addition of anti-OX40 to this therapeutic regimen lead to the formation of a positive-feedback loop composed of tumor antigen-specific T cells, IFN-γ and IFN-γ-induced chemokines in the tumor microenvironments. Based on our data, we conclude that the combination of dasatinib and anti-OX40 provides much better therapeutic efficacy than either drug alone and this combination gives a synergistic effect on the development of T cell-mediated anti-tumor immune responses. Our experiments delineate a mechanism by which this specific targeted therapy and immunotherapy can be combined to achieve a better anti-tumor therapeutic effect.

Key Words: Combination immunotherapy, Kinase inhibitor, Targeted therapeutics.

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EX VIVO EXPANSION OF PLACENTA DERIVED NATURAL KILLER CELLS FOR CANCER IMMUNOTHERAPY

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Human placenta derived stem cells (HPDSC) is a novel source of multipotential stem/progenitor cells obtained from full-term human placenta which is readily available and non-controversial. We have demonstrated that the donor-matched HPDSC and umbilical cord blood (UCB) are a rich source of placental immature NK (piNK) cells with unique properties of proliferation and cytotoxicity suggesting that piNK cells may provide a treatment option for a variety of cancers. Following the isolation from the cryopreserved donor-matched HPDSC and UCB units CD56+ CD3- piNK cells were culture expanded up to 3 weeks in the presence of IL-2 (200 IU/mL) and feeder cells K562 and peripheral blood mononucleated cells. This process yielded clinically relevant cell doses with an average of 1.2 billion cells per donor (n=6) that were >80% CD56+CD3- . The overall properties of expanded piNK cells were evaluated by extensive immunophenotypic studies and cytotoxicity assays. Overall our results suggest that from day 0 to day 21 the expression of NKG2D in 12 donors was increased from 60.9% ± 4.8% to 86% ± 17.4% (p value of 0.024); NKp46 was increased from 10.5% ± 5.4% to 82.8% ± 9.0% (p value of 0.00002); NKp44 was increased from 9.6% ± 6.5% to 51.6% ± 27.5% (p value of 0.022); and 2B4 was decreased from 13.0% ± 7.1% to 0.65% ± 0.5% (p value of 0.009%). Under these culture conditions the inhibitory KIRs including KIR3DL1 and KIR2DL2/L3 remained constant during 21-day expansion. The changes in the expression NKRs were further correlated with a marked increase in cytolytic activity at day 21 versus day 14 against K562 cells (63% ± 15% versus 45% ± 4%, p value of 0.0004). These findings demonstrated the high expandability of piNK cells and that the ex vivo expansion process can increase the in vitro cytolytic activity of piNK cells.

To gain insights on tumor cells susceptibility to cultured piNK cells, a wide range of tumor cell lines (n=12) were co-cultured with piNK cells. At effecter to target (E:T) ratio of 10:1, the cytotoxicity of day 21 expanded piNK cells towards U937 cells (lymphoma) = 89.2% ± 9.8%, WERI-RB-1 cells (retinoblastoma) = 73.3% ± 11.8%, RPMI8226 cells (multiple myeloma) = 61.3% ± 1.3%, and U266 cells (multiple myeloma) = 57.4% ± 4.7%. Furthermore, we have shown in NOD/SCID tumor xenograft models that the cytolytic activity of ex vivo expanded piNK cells correlates with in vitro activity.

In summary, we have demonstrated that piNK cells can be readily obtained, expanded, characterized and activated to yield a clinical relevant quantity of highly cytotoxic off-the-shelf cellular product for a wide range of hematological cancers.

Key Words: Adoptive therapy, Interleukin-2, NK cells.

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IDENTIFICATION OF IN VIVO-ACTIVATED MELANOMA-REACTIVE T CELLS BY HPRT SURROGATE SELECTION

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In vivo hypoxanthine-guanine phosphoribosyltransferase (HPRT)-deficient T cells (MT) from melanoma patients are enriched for in vivo clonally amplified T cells that traffic between blood and tumor tissues, and these MT have T cells with TCR beta chain (TRB) amino acid similarity to T cells with demonstrated melanoma-reactivity. Here we investigate MT as immunological probes for immunogenic tumor antigens in melanoma patients. MT were obtained by 6-thioguanine (TG) selection of lymphocytes from peripheral blood and tumor tissues, and wild-type T cells (WT) were obtained analogously without TG selection. MT from tumor-infiltrated lymph nodes (TILN) or from tumor-infiltrating lymphocytes (TIL) were significantly enriched for T cells binding melanoma antigen peptide MHC Class I multimerized reagents compared to WT (3 of 5) ($p < 0.001$). In addition, MT proliferated in response to dendritic cells pulsed with these peptides. Tumor specific T cells exhibit diverse functional profiles including those that do not follow the standard Th1/Th2 dichotomy {{178 Wang,H.Y. 2004; 825 Kyte,J.A. 2009; }}. We therefore developed an intracellular cytokine flow cytometry antibody panel to assess cytokine profiles that cross "traditional" T cell subset boundaries to monitor T cells from melanoma patients. MT recognized naturally processed epitopes on tumor cells, as the frequency of T cells positive for IFN-γ, TNF-α, IL-2, IL-4, or IL-10 following stimulation with irradiated melanoma cells was significantly greater than matched unstimulated T cells ($p < 0.01$). Furthermore, multi-parameter cytokine analysis demonstrated polyfunctional MT subsets that co-produce IFN-γ, TNF-α, and IL-2 or IFN-γ and IL-10. The present study suggests that HPRT surrogate selection may provide a mechanism to isolate and characterize T cells that are involved in the in vivo T cell response to melanoma.

Key Words: CD4+ T cells, CD8+ T cells, Cellular immunity.

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A PHASE II STUDY WITH THE ANTI-CTLA-4 mAb TREMELIMUMAB IN CHEMOTHERAPY-RESISTANT ADVANCED MALIGNANT MESOTHELIOMA: SAFETY, TOLERABILITY, CLINICAL AND IMMUNOLOGIC ACTIVITY

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Background: Anti-CTLA-4 monoclonal antibodies (mAb) are showing significant activity in different tumor types; however, no data are available in malignant mesothelioma (MM) patients (pts). We report preliminary results of a phase II, single Institution, study investigating safety, clinical and immunologic efficacy of the fully-human anti-CTLA-4 mAb tremelimumab as second-line treatment for MM pts who failed a platinum-based regimen.

Methods: On May 2011, 18 of the 29 foreseen MM pts, 8 stage III and 10 stage IV, 14 males, 4 females, median age 64 (47-76) years, ECOG performance status 0-2, were enrolled in the study and received tremelimumab at 15 mg/Kg i.v. on day (d) 1 and 90 for 4 cycles or until progressive disease (PD). Primary endpoint was objective response (OR); safety, disease control rate (DCR) (partial response (PR) + stable disease (SD), overall survival (OS), and immunologic activity were secondary. Tumor assessment at baseline, and at each cycle utilized modified RECIST Criteria. Adverse events (AE) were collected according to the Common Terminology Criteria v3.0. Peripheral blood mononuclear cells collected at baseline, d 14, 30, and 60 of the 1st and 2nd cycle, were analyzed by flow cytometry for an extensive panel of phenotypic and activation markers.

Results: All pts received at least 1 dose of tremelimumab (median 2; range 1-4). One patient (pt) achieved PR that evolved in persisting SD (+360 d); 4 pts had persisting SD lasting 270, 270, +180, +90 d, and 13 pts had PD. DCR was 27.8%. The median OS in the first 11 pts was 12,5 months (95% CI 9.4 -15.6), and the OS rates at 1 year 55%. Grade 1-2 AE occurred in 83% of pts; two pts had gastrointestinal grade 3 or hepatic and pancreatic grade 4 AE that resolved after steroids. Among investigated markers, a significant increase in the absolute number of CD4+HLA-DR+, CD4+CD45RO+, CD4+ICOS+, CD8+HLA-DR+, and CD8+ICOS+ T cells was detected at d 14, 30 and 60 after the first dose of tremelimumab, it slowly declined thereafter, and resumed upon re-dosing.

Conclusion: CTLA-4 blockade in MM pts is safe and shows promising clinical activity. A sizeable proportion of pts experienced clinical benefit, prolonged disease stabilization and extension of survival. Treatment associates with major changes in activated and memory T cell subpopulations.

Key Words: CTLA-4.

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IMPROVING MELANOMA TUMOR-INFILTRATING LYMPHOCYTE PHENOTYPE AND FUNCTION FOR ADOPTIVE CELL THERAPY THROUGH THE TNFR SUPER FAMILY MEMBER 4-1BB

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Adoptive T-cell therapy (ACT) is a powerful immunotherapy for stage IIIc-IV metastatic melanoma. ACT uses tumor-infiltrating lymphocytes (TIL) that can be expanded in two phases; phase one involves expanding TIL from excised tumor pieces with IL-2, followed by the phase two that involves a rapid expansion protocol (REP) where the TIL are activated with an anti-CD3 antibody in the presence of feeder cells and IL-2 over a 2 week period. CD8+ T cells have been found to be critical in mediating tumor regression during ACT. Although the REP yields billions of highly-differentiated CD8+ T cells, the post-REP TIL lose critical costimulatory molecules CD28 and CD27, are hyporesponsive to further proliferative signals and are sensitive to activation-induced cell death (AICD). We previously observed that CD8+ TIL losing CD28 up-regulate alternative costimulatory molecules of the TNF-R family, such as 4-1BB. In our previous study, we focused on the role of 4-1BB in post-REP CD8+ TIL and found that 4-1BB costimulation protected the CD8+ T cells from AICD and enhanced anti-tumor effector function. In our current study, we followed up on our initial observations and asked whether activation of the 4-1BB costimulatory pathway, using an agonistic anti-4-1BB antibody had an effect when added during the initiation of the TIL REP. We found 4-1BB costimulation increased CD8+ T-cell recovery and enhanced the anti-tumor killing activity of the TIL product. This was associated with increased expression of CTL-related factors Granzyme, Perforin, and Eomesodermin. This improved CTL phenotype was not associated with KLRG1 expression and CD28 loss. TIL receiving 4-1BB costimulation during the REP had higher bcl-2 gene expression and increased resistance to apoptosis. This was associated with longer persistence of the TIL in vivo following adoptive transfer into NOD-SCID x gamma c-/- (NSG) mice. Our findings suggest that augmenting TNFR costimulation through 4-1BB costimulation during melanoma TIL expansion significantly improves the phenotype and function of tumor-reactive CD8+ CTL, preserving certain memory T-cell properties without driving senescence. This approach may greatly improve TIL persistence and anti-tumor activity in vivo after adoptive transfer into patients.

Key Words: Adoptive therapy, Melanoma.

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IMMUNOLOGICAL AND BIOLOGICAL CHANGES AND THEIR CORRELATION WITH CLINICAL RESPONSE AND SURVIVAL DURING IPILIMUMAB IN METASTATIC MELANOMA COMPASSIONATE USE PROGRAM

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Backgroud: Recently FDA has approved Ipilimumab at 3mg/kg as first and second line of therapy in patients with metastatic melanoma. This is sustained by an impact on overall survival in this setting of patients of 10.1 months. Anyway no clinical parameter has been consistently found to be a surrogate or a predictive marker for response to ipilimumab therapy and only a few immunologic changes (absolute lymphocytes value) have been demonstrated.

Patients and Methods: From June 2010 to date we have been treating in the Compassionate Use Program for ipilimumab at 3 mg/Kg fifty pretreated metastatic melanoma patients. 35 out of 50 patients (70%) completed all four doses and were considered evaluable for clinical response, toxicity and sieric changes of LDH and RCP (reactive C protein), and time to progression (TTP). For RCP evaluation we defined 3 categories: < 5 mg/dl for normal values, ≥5<8 for high values and ≥ 8 to indicate very high values. According the immunological and biological assessment we have collected PBMC and sera of these patients. Blood draw was performed at week 0, 4, 7, 10 and 12. PBMC were thawed and labeled with FoxP3-AlexaFluor488/CD4-PE-CY/CD25-PE (Kit Biolegend). Labeled cells were analyzed using a FACSAria II (Becton Dickinson). We have also studied serum cytokines (IL-10, IL-6 and TGF-b) and auto-ab (as Anti DS-DNA, Anti-Tg, ANA), that were measured using enzyme-linked immunosorbent assay.

Results: In this setting of patients, we found in 30/35 (85%) of them a good correlation between the increase of LDH and CRP, and the worsening of clinical response. For patients [17/35(48%)] with a rapid progressive disease not responsive to ipilimumab, we found that the percentage of Treg increased during the treatment (median: 1.8%; range 1% -2.6%); this increase was not influenced by development of autoimmunity. In this responsive patients group [18/35(51%)] the values of Treg remained stable at 0,50% [(10/18 (55%)], while the remaining group [8/18(45%)] decreased of 0,10% per cycle. At moment, no changes in sieric cytokines and antibodies have been found.

Conclusion: LDH and RCP seem to be predictive parameters of response to ipilimumab. Moreover, very preliminary data shows a relationship between the increase of the circulating Treg cell percentage and a bad response to ipilimumab. Further studies are necessary to verify this data.

Key Words: Ipilimumab, Melanoma, Treg cells.

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ANTI-CCL2 MONOCLONAL ANTIBODY THERAPY OF MAMMARY CARCINOMA

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CCL2 (Monocyte Chemoattractant Protein-1 (MCP-1)) is a chemokine that has potent mononuclear cell chemoattractant, activation and proliferative properties as well as playing a role in tumorigenesis. In experimental models and cancer patients, myeloid derived suppressor cell (MDSC) numbers increase with tumor progression, both in the periphery and at the site of tumor and are responsible for suppressing host immune responses and directly promoting tumor growth. CCL2 is produced by tumor-induced MDSC and many tumor cells, including the mammary carcinoma cell lines AT-3 (C57BL/6) and 4T1 (BALB/c). Given the role of CCL2 in cancer pathobiology and the roles of MDSC in cancer progression and immunosuppression, targeting CCL2 is a logical avenue of investigation. In the 4T1 and AT-3 models of breast cancer we demonstrate that anti-CCL2 mAb therapy can be used to reduce both primary and metastatic tumor growth. This reduction in tumor growth correlated with a decrease in the accumulation of peripheral splenic MDSC and in the proportion of tumor-associated macrophages (TAM) and MDSC at the site of tumor, as well as a reduction in tumor vascularization. The efficacy of anti-CCL2 therapy was dependent to some extent on the route and timing of therapy delivery. The role of CCL2 in tumor progression may act in a biphasic manner, such that administration of anti-CCL2 early after tumor implantation resulted in an initial enhanced tumor growth, whereas administration of anti-CCL2 to mice with established tumors, led to tumor growth inhibition. We hypothesize that there was an early inhibition of immunosurveillance, but then once a tumor had established, the CCL2-mediated attraction of immune cells such as TAM and MDSC to the tumor site had a pro-tumourigenic effect that was inhibited by the anti-CCL2-mediated reduction in the migration of these cells. Evidence for the therapeutic potential of anti-CCL2 mAb therapy justifies the further characterization of the role of CCL2 in tumor progression and its potential as an adjuvant for other cancer immunotherapies.

Key Words: Breast cancer, Chemokines, Myeloid derived suppressor cell.

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PHASE I STUDY OF THE ANTI-CD22 IMMUNOTOXIN MOXETUMOMAB PASUDOTOX (CAT-8015 OR HA22) IN PEDIATRIC PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) - UPDATED RESULTS

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Background: New therapies are needed to overcome chemotherapy resistance and reduce toxicities for patients with ALL. CD22 is a B-lymphoid differentiation antigen expressed on most ALL blasts. We are conducting a Phase I trial of a high-affinity anti-CD22 immunotoxin, moxetumomab pasudotox.

Design: Patients with relapsed/refractory ALL receive moxetumomab pasudotox at 5, 10, 20, or 30 mcg/kg IV QOD for 6 doses every 21 days. An initial completed cohort (A) included one patient each at the first 3 dose levels followed by standard 3+3 dose escalation commencing at 30 mcg/kg. In attempt to reduce vascular leak syndrome (VLS) incidence, an ongoing cohort (B) also receives dexamethasone 2.5 mg/m2 every 12 hours around moxetumomab pasudotox doses during cycle 1; standard 3+3 dose escalation commenced at 20 mcg/kg.

Results: Currently, 14 patients aged 5-21 years (median, 11) with ALL (13 precursor-B, 1 mature B-cell) have been treated. Patients had a median of 4 prior regimens (range, 2-8), 12 were chemotherapy refractory, and 8 had prior stem cell transplantation. Thirteen with leukemia-associated baseline cytopenias were not evaluable for hematologic toxicities. Most common adverse events to date: decreased neutrophil, leukocyte and platelet counts, transaminase elevations, decreased hemoglobin, abdominal pain, hypoalbuminemia, pyrexia, tachycardia, elevated creatinine, febrile neutropenia, hypokalemia, hypoxia. Dose-limiting toxicity VLS (Grade 3 or 4) occurred in 2/7 cohort A patients (30 mcg/kg) and 0/7 cohort B patients. All drug-related toxicities were reversible. Twelve patients were evaluable for response: 3 (25%) had complete responses; 6 (50%) had hematologic activity (blood count improvement, blast reduction); 2 (17%) had stable disease; and 1 (8%) had progressive disease. Two patients developed high-titer neutralizing antibodies.

Conclusions: Moxetumomab pasudotox is active against chemotherapy-refractory pediatric ALL, with complete remissions achieved in 3 of 12 patients. Based on the clinical activity and safety profile, moxetumomab pasudotox warrants further study in ALL.

Sponsored by MedImmune, LLC.

Key Words: Leukemia, Phase I, Targeted therapeutics.

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LOW EXPRESSION OF CXCR3 AND CCR5 CHEMOKINE RECEPTORS IN TUMOR-INFILTRATING LYMPHOCYTES IS ASSOCIATED WITH FAVORABLE OUTCOME 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 and CCR5-ligand chemokines is critical for the development of immune-mediated rejection.

We previously showed that co-down-regulation of CXCR3 and CCR5 genes in tumor-infiltrating lymphocytes (TIL) is predictive of complete remission (CR) to adoptive therapy. However, patients carrying the CCR5 Δ32 (a deletion of 32 bases encoding a protein not expressed on cell surface) are expected to have a reduction of CCR5 protein expression (i.e., -50% in heterozygous and -100% in homozygous patients). Here, we developed a protein prediction model (PPM) by integrating DNA and RNA data. The PPM was validated by flow-cytometry (FACS) analysis.

Methods. One-hundred-forty-two TIL samples, belonging to 142 melanoma patients enrolled in consecutive adoptive therapy trials were evaluated. Genotyping (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). PPM model was validated by FACS analysis (50 TIL samples including 15 CR, 6 PR and 29 NR). The patients with CXCR3CCR5 gene-expression values below the median level were included in the CXCR3CCR5-low group. In the PPM model CCR5Δ32 carriers with high CCR5 and low CXCR3 transcript levels were included in the CXCR3CCR5-low group.

Results. The gene expression model was predictive of CR [(CR:29% (12/39) vs. 4% (5/103), CXCR3CCR5 low vs other patients, respectively (P=.00007)] but not of overall response (OR). Twenty-five CCR5Δ32 were found (heterozygous, N=24; homozygous, N=1). By adding the DNA dimension to the RNA data, the PPM was more accurate in predicting CR (P=.00004) and was also predictive of OR [OR:64% (30/47) vs. 43% (41/95); CXCR3CCR5 low vs other patients, respectively (P=.00007)]. FACS analysis validated the PPM (gene-protein expression Spearman's correlation ρ=0.68, P=.000001 and ρ=0.44, P=.001, for CCR5 and CXCR3, respectively). Both gene expression model and PPM were predictive of CR in multivariate analysis with other clinical parameters.

Conclusions. Low expression of CCR5 and CXCR3 chemokine receptors in TIL is associated with CR and 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.

Key Words: Cytokine, Melanoma, Tumor infiltration lymphocytes.

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CONVOLUTING THE PANCREATIC TUMOR MICROENVIRONMENT: RESPONSE TO PLATINS

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Objectives/Background: Emergent tumors are complicated mixtures of tumor cells, vessels, supporting and inflammatory cells. We plan to reconstitute the 'de-convoluted' tumor microenvironment (TME) cell-by-cell in tissue culture and examine the response to various therapeutic strategies.

Methodology: We mixed tumor cells and fibroblasts, allowing them to grow in culture together for varying time periods. Tumor cells grow as small E-cadherin+ "nests" within large fibroblast swaths. Gemcitabine, Oxaliplatin and Cisplatin were used to treat the tumor cells alone, co-cultures or fibroblasts alone for 2 and 24 hours. Mouse embryonic fibroblasts derived from HMGB1 KO mice and WT littermates were used.

Results: Increasing the gemcitabine concentration up to 3 mg/ml, tumor cells have an IC50 of 0.01 μg/ml while fibroblasts alone are much less sensitive (IC50 of 925 μg/ml). The co-culture didn't affect the fibroblasts, but did increase the IC50 of the tumor cells up to nearly 1 μg/ml. With platins on the other hand, survival of the fibroblasts diminished in the co-cultures while tumor cells behaved comparable to being alone. Cisplatin treatment up to 10 mg/ml gave IC50s of 100 μg/ml for cancer and 2 mg/ml for fibroblasts alone. When cells were cultured together, the IC50 shifted for both cells to around 50 μg/ml.

Significance: Classic cancer therapeutic development has only studied the tumor cell, leaving out several essential components of the TME. In this preliminary study, we reconstituted some of the components of the TME to determine the requirements for stromal support.

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

Presenting author underlined. *Primary author in italics.*

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BIDIRECTIONAL INTERACTIONS OF TUMOR AND THE MICROENVIRONMENT: LEUKEMIA CELL LINES INDUCE CHANGES IN BONE MARROW STROMAL CELLS

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Background: Bone marrow-derived stromal cells (BMSC), or mesenchymal stem cells (MSC), are non-hematopoietic precursor cells residing in the bone marrow. BMSC support vasculogenesis and wound healing. In the bone marrow, BMSC are involved in the regulation of hematopoiesis: they keep the dormant hematopoietic stem cells (HSC), at the endosteal niche, and they play a role in the release of activated HSCs at the perivascular niche. BMSC show a complex number of immunomodulatory effects on T-cells, B-cells, DC and NK cells. BMSC are also attracted to the primary and metastatic tumor environments, where they become tumor-associated fibroblasts, thus affecting tumor survival and angiogenesis. BMSC secrete a wide range of cytokines, growth factors and matrix proteins that are likely responsible for many of these effects. We hypothesized that the tumor may affect the type and quantity of cytokines and growth factors produced by BMSC. Using leukemia as a model, we investigated the effects of leukemic cell lines on BMSC.

Methods: Three different Leukemia cell lines (TF-1, TF1α and K562) and CD34+ cells (HSC) from three controls were co-cultured with BMSC from healthy donors using a Transwell system (1µ pore size). Cells and supernatants were harvested after 4h, 10h, 24h, 3 and 5 days. BMSC, Leukemia and CD34+ cells were used for gene expression profiling using a Whole Human genome Oligo Microarray Kit (Agilent). The proliferation status of BMSC and leukemic cells were analyzed after 3 and 5 days of co-culture.

Results: Co-culture resulted in changes in leukemia cell line gene expression profiles. Surprisingly, the leukemia cell lines induced BMSC gene expression profile changes, and the BMSC reacted differently to the 3 different leukemia cell lines and CD34+ control cells. When cocultured with TF-1α, the cell lines with less mature features, BMSC showed changes in expression of genes related to self-renewal pathways (Wnt pathway). After co-culture with TF-1 and K562 cells, BMSC showed an increased expression of pro-inflammatory related genes (IL8, CXCL1, CCL2 and IL17 signaling related genes). Co-culture of BMSCs with CD34+ cells resulted in far fewer changes; the BMSC genes that did change were related to metabolic pathways. BMSC proliferation decreased when they were co-cultured with TF-1 and K562 cells, but not when co-cultured with TF-1α cells.

Conclusion: The interactions between leukemia and stroma are bidirectional. BMSC cultured with some, but not all, leukemic cell lines showed an increased expression of genes related to pro-inflammatory pathways, particularly IL-17, and a decreased proliferation activity.

Key Words: Cytokine, Leukemia, Tumor microenvironment.

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INDUCTION OF MART-1 CD8+ T CELLS IN THE TUMOR MICROENVIRONMENT FOLLOWING VACCINATION WITH AN ONCOLYTIC HERPES VIRUS ENCODING GM-CSF (ONCOVEX)

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Introduction: OncoVEX is an attenuated oncolytic herpes virus encoding human GM-CSF. The mechanism of anti-tumor activity is related to the oncolytic effects of the virus as it can only replicate in tumor cells and the induction of local and systemic immunity. We sought to confirm the hypothesis that vaccination with OncoVEX could induce local and systemic anti-tumor immunity in a cohort of patients with metastatic melanoma treated on a clinical trial of OncoVEX.

Methods: Patients with accessible metastatic melanoma were enrolled in a single arm open-label Phase II (N=50) or a prospective randomized Phase III (N=439) trial. All patients assigned to OncoVEX treatment received an initial dose of 1 x 106 pfu/ml virus followed three weeks later by a dose of 1 x 108 pfu/ml repeated every two weeks until disease progression or complete response. In selected patients, tumors were resected or underwent biopsy to confirm a pathologic response or for palliation. T cells were extracted by Ficoll separation and subjected to analysis by flow cytometry, interferon-gamma ELISPOT or dextramer analysis in HLA-A2+ patients. Where practical, PBMC were also collected and analyzed in a similar manner.

Results: Analysis of the tumor microenvironment revealed the induction of MART-1-specific, but not gp100- or tyrosinase-specific, CD8+ T cells by dextramer analysis. The functional status of these cells was confirmed by ELISPOT analysis. The magnitude of the CD8+ T cell response was greatest in vaccinated lesions but MART-1-specific CD8+ T cells could be detected at lower frequency in non-injected lesions and peripheral blood from the same patient. There were also a significantly decreased number of CD4+FoxP3+ T cells in OncoVEX injected tumors compared to non-injected control patients.

Discussion: OncoVEX induces local and systemic MART-1-specific T cell immunity in patients with metastatic melanoma consistent with its proposed mechanism of action.

Key Words: Cancer vaccine, CD8+ T cells, Tumor microenvironment.

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INCREASED POLYFUNCTIONAL CYTOKINE EXPRESSION AND PROLIFERATION OF CD4+ T CELLS IN HEAD AND NECK CANCER PATIENTS IS ASSOCIATED WITH THE ELEVATED B-CELL FREQUENCY AFTER RADIO-CHEMOTHERAPY

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Background. Standard treatment of head and neck squamous cell cancer (HNSCC) includes radical surgery and various regimen of radio-chemotherapy (RCT). However, median survival remains low mainly due to recurrent disease, and it has been suggested that dysregulation of anti-tumor responses in HNSCC patients contributes to poor outcome. The role of RCT in reversing immune dysregulation is unknown.

Methods. Peripheral blood samples were obtained from normal donors (NC, n=20) and patients with HNSCC (n=60). The frequency of CD19+ B-cells and CD4+ T-cells was monitored by flow cytometry. Polyfunctional cytokine expression in CD4+ T cells was measured by ten-color flow cytometry in co-cultures with B-cells isolated by magnetic beads from PBMC of NC and HNSCC patients. Proliferation was measured in CFSE-based assays.

Results. After RCT, HNSCC patients had an altered CD4+ T to B cell ratio: the frequency of CD4+ T-cells was low and that of B-cells was high compared of patients prior to treatment or to NC ($p < 0.05$ for both). The frequency of CD4+ T-cells positive for IL-6, IL-2, IFN-γ, and TNF-α, as well as the number of cytokines expressed per T-cell was increased in HNSCC patients after RCT ($p< 0.05$). Proliferation and cytokine expression in isolated CD4+ T cells was enhanced in the presence of CD19+ B-cells ($p< 0.05$).

Conclusion. RCT induces T-cell activation and expression of cytokines in T helper cells. It also alters the ratio of T to B-cells potentiating immune responses. These changes in tissues and blood may be responsible for beneficial effects of RCT in this disease.

Key Words: B cell, CD4+ T cells, Cytokine.

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A CCR4 ANTAGONIST COMBINED WITH TUMOR VACCINES EFFICIENTLY BREAKS TOLERANCE, ELICITS CD8+T CELLS AND ANTI-TUMOR IMMUNITY

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Regulatory T cells (Treg) may impede vaccine efficacy in cancer. CCR4 antagonists, an emergent class of Treg inhibitor, have been shown to block recruitment of Treg into lymph node mediated by CCL17 and CCL22. As most tumor antigens are self antigens possibly controled by Treg, our aim was to demonstrate the ability of a CCR4 antagonist to induce CD8+T cells directed against various self tumor antigens. For this purpose we selected various transgenic mice expressing Her2/neu, E7 or OVA as self antigen. Protein based vaccine vectorized or not by the B subunit of Shiga toxin (a vector targeting dendritic cells) and a DNA based vaccine coding for the E7 protein derived from HPV were tested in combination or not with the CCR4 antagonist. Induction of functional anti-self CD8+T cells could be observed against various model self antigens (Her2/neu, E7, OVA) only when protein (delivered via the B subunit of Shiga toxin) - or DNA-based vaccines were combined with the CCR4 antagonist. Antigen specific CD8+T cells were detected by Tetramer and Elispot assays. This strategy to block Treg was more efficient than cyclophosphamide and similar to the depletion of Treg by anti-CD25 mAb. However compared to mAb, the CCR4 antagonist has a short life time which may avoid potential autoimmune complication caused by long term blockade of Treg. These vaccines combined with the CCR4 antagonist were also more efficient than the vaccine alone to inhibit the growh of tumors expressing self antigen.

As only 20% of Treg in mice expressed CCR4, we further characterized this population and showed that it corresponded to memory (CC44high) activated (ICOS+) cells. The efficiency of the CCR4 antagonist strongly suggest that these CCR4+Tregs represent an important population to be targeted to modulate T reg activity. In human, we showed that CCR4 is expressed by more than 70% of peripheral or intra-lymph node Treg. The previous demonstration that a CCR4 antagonist is efficient to block human Treg, together with the high expression of CCR4 in human Treg also provide some rationale to develop this new class of Treg inhibitor in human. Our vaccine combining an efficient antigen delivery system which targets dendritic cells (the B subunit of Shiga toxin) to a CCR4 antagonist able to break tolerance mediated by Treg during the priming phase may thus represent a prototype cancer vaccine to elicit potent functional anti-tumor CD8+T cells in the context of immunosuppression mediated by Treg in cancer patients.

Key Words: Cancer vaccine, Chemokines, Treg cells.

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IL-18-INDUCED NK HELPER CELLS MEDIATE THE ATTRACTION AND ACTIVATION OF DCS, PROMOTING THE ACCUMULATION OF TYPE-1 EFFECTOR T CELLS

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The chemokine-driven association of immune cells critical to the initiation and propagation of immune responses is essential for the development of effective immunity against cancer. We have previously demonstrated that natural killer (NK) cells can be activated, through exposure to IL-18, to a unique 'helper' differentiation pathway distinct from 'effector/killer' differentiation. These IL-18-induced 'helper' NK cells are capable of synergizing with secondary signals, including type I interferons, to promote dendritic cell (DC) activation and the development of DC-mediated type-1 immune responses. Here, we demonstrate that such IL-18-induced 'helper' NK cells produce high levels of immature DC (iDC)-attracting chemokines, including CCL3 and CXCL8, and functionally attract iDCs in a primarily CCR5-dependent mechanism. We also describe the unique ability of these 'helper' NK cells to prime DCs for high production of CXCR3 and CCR5 ligands, facilitating the additional recruitment of type-1 effector T cells (CTLs). This study demonstrates unique chemokine regulation of 'helper' versus 'effector' pathways of NK cell differentiation in the initiation and propagation of DC-mediated immune responses. These data further provide rationale for the therapeutic use of properly-activated NK cells in promoting the development of type-1 immune responses and the enhancement of DC-based vaccines.

Key Words: Chemokines, Dendritic cell, NK cells.

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CHANCE AND CIRCUMSTANCE GOVERN MACROPHAGE FUNCTIONAL POLARIZATION

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Macrophages play a critical role in maintaining the balance between homeostasis and protective inflammation by functionally polarizing towards either immunostimulatory (M1) or immunosuppressive (M2) phenotypes. Tumors manipulate this balance via immunosuppressive stimuli (e.g., IL-10), shifting tumor-associated macrophages (TAMs) to a M2 phenotype that promotes tumor survival and poses a barrier to therapeutic intervention. We seek to understand the macrophage polarization process and identify novel strategies for enhancing the efficacy of cancer immunotherapies. Here, we describe several novel modalities by which this important process is controlled.

Macrophage polarization is plastic; it has been shown that TAMs can be converted from an M2 phenotype to an M1 phenotype via the application of stimuli such as interleukin-12 (IL-12). However, investigations to date have focused on the application of coherent signals (either pro-M1 or pro-M2 stimuli alone) even though immunostimulatory and immunosuppressive stimuli rarely exist independently in vivo, especially at tumor sites. How innate immune cells "calculate" a response to contradictory signals was unknown. To address this question, we polarized macrophages using various dose combinations of both IL-10 and IL-12, and then "activated" the cells with lipopolysaccharide (LPS), which enhances M1- or M2- type responses in correspondingly polarized cells. Cellular responses were assessed via quantitative gene expression profiling. Surprisingly, the presence of IL-10 prevented IL-12-mediated promotion of an M1 phenotype. Moreover, M2-type responses increased with IL-10 dose and were largely independent of IL-12 co-treatment.

Because individual cells may respond differently to identical stimuli, we next examined macrophage polarization at the single-cell level. We first analyzed cells polarized under the conditions described above by flow cytometry, which revealed M1 and M2 cells co-existing within single cultures. The probability of polarization towards an M2 state increased with IL-10 dose, and this probability was independent of IL-12 co-treatment. Interestingly, some cells remained non-responsive to LPS-mediated activation, and this probability of activation was independent of polarizing cytokine treatment. This is the first evidence that macrophage polarization is stochastic, and it suggests that separate stochastic regulatory processes govern activation and polarization. Elucidating these processes, as well as the mechanism by which IL-10 dominates macrophage polarization, should help to identify novel therapeutic targets and strategies for treating cancer and diseases of chronic immune dysfunction.

Key Words: Innate immunity, Macrophages, Tumor microenvironment.

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IMMUNE THERAPIES FOR CANCER: BIMODALITY - THE BLIND SPOT TO CLINICAL EFFICACY - LOST IN TRANSLATION

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Multiple attempts to improve the clinical success rates of cancer therapies, including immunotherapies, show variable, confusing results, with few complete responses, the remainder being PR, SD and progressive disease. It is widely assumed that observed response heterogeneity - a feature almost independent of therapy type (cytotoxic chemotherapy, radiation, biological, pathway blocker, antibody, or vaccine) - is due to resistant cancer cell clones, antigenic variations and/or poor antigen presentation. Lack of research progress suggests antigenic variability, defective tumor antigen presentation, and even cytotoxic resistance are probably not main causes. T-cell immune responses can be detected in most, if not all, cancers indicating on-going underlying pre-exisiting endogenous immune response occurs in most cancer patients, but is rendered ineffective by 'skewing' the immune response balance towards a regulatory, rather than effector, dominance. Recent literature strongly implicates predominant T-regulatory response as the most likely culprit for in-vivo immune response clinical ineffectiveness, in most tumor types.

Emerging evidence shows certain cytokine receptors are transiently bimodally expressed on both effector and regulatory cells, rather than on one cell type alone, as previously thought. The net effect of cytokines, acting through the specific receptor (eg. IL2/IL2R) is determined by numbers of receptors expressed on a particular cell type at the time of exposure to the cytokine. Moreover, the relative predominance of cytokine receptor expression - either on effector or regulatory cells - would be expected to determine the direction the immune response is driven (responsiveness or tolerance) at the time of therapy.

The time of administration of the agent (cytokine, vaccine, CTLA4 inhibitor, antibody or even cytotoxic) would therefore be expected to determine the intensity, direction and clinical efficacy of the respective therapeutic agent and would explain the heterogeneous mix of complete and partial responses and failure to respond. It would also explain how repeat therapies sometimes produce successful clinical results, where they did not initially. Repeated frequent therapies, and combined chemo-immuno therapies could also be explained using this reasoning. Pre-conditioning therapies might optimize the probability of successful therapeutic immune responses. All of these are predicated upon the most optimal timing of therapies to engineer more effective clinical responses. Reported cyclical behaviour of the immune system would explain the paradoxical observations for most therapies and lack of progress to date.

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

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BLOCKING B7-H4-MEDIATED T-CELL INHIBITION TO ALTER THE TUMOR MICROENVIRONMENT

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B7-H4 is a member of the B7 superfamily that negatively regulates T cell proliferation and activation. While B7-H4 expression on tumor cells is associated with adverse prognosis in cancer patients, B7-H4 expression on tumor-associated macrophages (TAMs) characterizes a potent immunosuppressive population of the tumor microenvironment that correlates significantly with the number of tumor-infiltrating T regulatory cells (Treg). However, B7-H4 function during the cross-talk between tumor cells and tumor-infiltrating immune cells is not known.

Our study explores the soluble interactions between tumor cells and the surrounding microenvironment. We hypothesized that B7-H4 expression on TAMs facilitates tumor immune evasion in a T-cell dependent manner by promoting a suppressive microenvironment. Blocking B7-H4-mediated T-cell inhibition could alter the tumor microenvironment and inhibit tumor growth. Our research objectives were to study the ovarian cancer-related molecular mechanisms that regulate B7-H4 expression in TAMs.

We characterized B7-H4 expression at the cell surface of ovarian cancer cells from ascites and solid ovarian tumors. We confirmed that B7-H4 was expressed by monocytes from ovarian cancer samples. Furthermore, to assess B7-H4 function in the tumor microenvironment we set up an in vitro model system of co-culture in-transwells using human macrophages and ovarian cancer cells (Ovcar3) and we generated anti-B7-H4 recombinant antibodies (scFv) by screening a novel yeast-display scFv library derived from ovarian cancer patients.

We found that B7-H4 expression on macrophages was up-regulated during in vitro transwell co-culture with tumor cells to compare with cytokine-maturated macrophages. In addition, in vitro generated TAMs could inhibit T cells proliferation and activation in a B7-H4-dependent manner. The anti-B7-H4 scFv (clone 26) could block B7-H4-mediated T cell suppression by macrophages and promote T cell proliferation and cytokine release.

In conclusion, our results indicate that utilizing anti-B7-H4 scFv to target B7-H4 on macrophages or tumor cells give insight into the B7-H4 activity on T cells and that interfering with B7-H4 signaling can activate anti-tumor immunity. Blocking B7-H4-mediated suppression of T cells could significantly improve immune recognition of tumors.

Key Words: Tumor microenvironment.

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PD-1 MEDIATED REGULATION OF T CELL DYSFUNCTION IN OVARIAN CANCER MICROENVIRONMENT

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Ovarian cancers are armed with potent signals that induce T cell exhaustion (1-3). Here we tested the role of programmed death-1 (PD-1) and its ligands, PD-L1 and PD-L2 in modulating key antitumor functions (2-3). We show that i) human ovarian tumors co-expressed high levels of PD-L1 and PD-L2; ii) human ovarian TILs expressed high levels of PD-1 and showed exhausted phenotype and inability to differentiate into memory cells; iii) in vitro disruption of PD-1 signaling enhanced the proliferative capacity and function of human ovarian Ag-specific T cells; iv) in vivo PD-1 pathway blockade expands TILs and thereby enhances regression of a syngeneic mouse model of ovarian cancer (ID-8); v) vaccination with ID-8 tumors expressing either GM-CSF or Flt3-ligand, TLR agonists, or anti-CD137 agonistic antibody adds value to PD-1, PD-L1 or PD-L2 blockade in ID-8 ovarian mouse model; and v) PD-1 pathway blocking antibodies cause regression of human ovarian tumors (OVCAR5) in vivo in a humanized mouse model system (HLA-A2+ NSG mice reconstituted with human CD34+ cells) (4); In addition, we assessed comparative efficacy of PD-1, PDL1 or PD-L2 blocking antibodies in either mouse model or in human ex vivo. Our study indicates that PD-1 and its ligands participate in a network of pathways that modulate T cell dysfunction and blockade of this pathway expands tumor-reactive T cells and drives ovarian tumor rejection.

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Key Words: Ovarian cancer, PD-1, Tumor microenvironment.

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mTERT VACCINE CHEMOTHERAPY COMBINATION AUGMENT ANTIGEN-SPECIFIC IMMUNE RESPONSE AND CONFER TUMOR PROTECTION

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Successful cancer chemotherapy relies on the comprehensive tumor cell elimination. However, at clinically tolerated doses, chemotherapeutic drugs usually fail to kill all tumor cells in vivo. Theoretically, to achieve complete eradication, partial tumor killing by chemotherapy should be accompanied by a "bystander effect" in which the immune system recognizes, attacks, and eradicates residual tumor cells. Doxorubicin (or adriamycin) is an anthracycline antibiotic that intercalates with DNA, inhibiting its replication. It has been demonstrated upregulated ability of doxorubicin treated dendritic cells to present antigen to Ag-specific T cells in vitro and also upregulation of the Ag-processing machinery (APM) proteins and costimulatory molecules. Pegylated liposomal doxorubicin (Doxil) extravasates efficiently through the leaky tumor vasculature and is protected from renal clearance, enzymatic degradation, and immune recognition, enhancing drug pharmacokinetics, reducing hematologic effects and achieving targeted delivery to the tumor site. The telomerase reverse transcriptase, TERT, is an attractive target for human cancer vaccination because its expression is reactivated in a conspicuous fraction of human tumors. Critical to the success of combination therapy using TERT vaccine and chemotherapy drugs is a positive (synergistic) interaction between chemotherapy drugs and immune effectors mechanisms. Indeed, activation of antitumor immune responses by vaccine could be antagonized by chemotherapy, if select chemotherapy drugs develop immune antagonistic interactions. Understanding the dose and the schedule of administration of Doxil it is critical for the design of rational combinations of vaccine therapy with chemotherapy. To test the interactions within TERT vaccine and Doxil we performed a series of experiment using four different therapeutic schedule Doxil/TERT administrations. Interesting we found that two of the four schedule of Doxil/TERT vaccine combination resulted in a significant tumor growth delay which was accompanied by also significant increase of anti-TERT CMI. Tumor microenvironments study also showed Doxil time dependent cytokine composition. This data unveil new facets "Chemo immune adjutancy" of Doxil/Doxorubicin demonstrate important in vivo effects of the combination and underline the importance of the timing in the administration of the vaccine and the chemotherapeutic drug.

Key Words: Chemotherapy, Combination immunotherapy, Tumor microenvironment.

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ENFORCED M1 POLARIZATION OF TUMOR MACROPHAGES INCREASES THE EFFICACY OF RADIATION THERAPY AND CHEMOTHERAPY

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By causing the death of cancer cells, radiation therapy and chemotherapy have been proposed to provide both tumor antigen and endogenous immune adjuvants to initiate de novo tumor-specific immune responses. However, following radiation therapy and chemotherapy of tumors in mice there is a rapid influx of macrophages that polarize towards the M2 phenotype of tissue repair and immune suppression. We demonstrate that these new macrophages upregulate Arginase I and VEGF and that the influx of M2 macrophages precedes re-establishment of angiogenesis in the tumor and tumor recurrence. We propose that polarization of macrophages to an M2 phenotype causes repair and recurrence following cytotoxic therapy. Therefore, we propose that redirecting macrophages to an M1 phenotype will prevent recurrence from residual disease. Using an in vitro model, we have identified that macrophage exposure to dying cancer cells causes M2 polarization, and using luciferase reporter assays we identified that this is a transcriptional switch consistent with an increasing contribution of NFκB p50 homodimers. Using bone marrow-derived macrophages from NFκB1 knockout mice we confirm that the angiogenic and immune suppressive polarization of macrophages following exposure to irradiated cancer cells requires NFκB p50. Importantly, we demonstrate that in vivo radiation therapy and chemotherapy are significantly more effective in NFκB1 knockout mice, resulting in improved control of disease and metastases. We propose that wound repair following cytotoxic therapy limits control of residual disease in part by increasing immune suppression in the tumor. Directing the polarization of newly recruited tumor macrophages following radiation therapy represents a strategy to enhance adaptive immune control of residual cancer cells.

Key Words: Chemotherapy, Macrophages, Tumor microenvironment.

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DISTINCT STATES OF IMMUNE SUPPRESSION IDENTIFIED USING CLUSTERED PHENOTYPES OF CANCER PATIENTS

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Immunosuppression is common in cancer patients and may play a significant role in its pathogenesis. Our goal is to identify and quantify immunosuppression using a global and systems based ap-

proach. The immune suppressive status of a patient is made up of an immune profile consisting of quantity (cells/ul) and ratios of each category of immune cells. To establish these profiles we have characterized the blood of cancer patients using flow cytometry to identify up to forty different circulating immune cells. We have applied a bioinformatics approach to this data in an attempt to reduce the dimensionality of information and to cluster like profiles. Our hypothesis is that there are multiple types of immune suppression seen in cancer and that these types may not be exclusive to the underlying tumor diagnosis. Thus, profiles that may be shared across tumor types may benefit from similar immune modulation or have similar prognosis. We used immunophenotypic data to perform unsupervised hierarchical agglomerative average linkage clustering and principal component analysis on data encompassing parameters obtained from newly diagnosed or recently relapsed patients diagnosed with glioblastoma, renal cell carcinoma (RCC), non-Hodgkin's lymphoma (NHL), pediatric sarcoma, or ovarian cancer. In total this analysis represents 109 cancer patients and 41 healthy volunteers. In a preliminary analysis of RCC, GBM, and NHL patients, we found that in group A, 5 cancer patients clustered with 17 healthy volunteers, in group B, 15 patients with 20 healthy volunteers, in group C, 32 cancer patients with 4 healthy volunteers, and in groups D and E , 20 and 7 cancer patients respectively. Differences in groups were related to changes in granulocytes, monocyte subsets (particularly suppressive monocytes), and lymphocyte subsets (in particular CD4 subsets). Detailed profile descriptions of the groups will be presented. With these data, we have used a bioinformatics approach to identify common multifactoral immune profiles in patients with cancer.

Key Words: Cellular immunity, Lymphoma, Ovarian cancer.

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DISTINCT REGULATION OF THE FUNCTION OF SUBSET OF MYELOID DERIVED SUPPRESSOR CELLS BY IFN-GAMMA

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The immune regulatory role of myeloid derived suppressor cells (MDSC) has been described in chronic inflammation and cancer. Recently, others and we have identified two phenotypically and functionally different subsets of MDSC, namely monocytic (CD11b+Gr-1dull/int.) and granulocytic (CD11b+Gr-1high) cells. These subsets utilize different effector molecules and signaling pathway in suppressing antigen-specific immune response. However, little is known on how the function of MDSC subset is regulated by IFN-gamma and details about the mechanism of such regulation is not available.

We found that CD11b+Gr-1high granulocytic MDSC have a higher expanding potential that CD11b+Gr-1dull/int. cells in tumor bearing mice. We further looked at the frequency of subsets in wild type and IFN-gamma R-/- mice. The proportion of CD11b+Gr-1high and CD11b+Gr-1dull/int. MDSC in tumor bearing IFN-gamma R-/- mice were comparable to that observed in wild type tumor bearing mice. However, depletion of IFN-gamma in co-cultures of monocytic MDSC with antigen specific splenocytes results in complete reversal of suppression by CD11b+Gr-1dull/int. MDSC where as CD11b+Gr-1high MDSC suppressed antigen specific immune response significantly only in the absence of IFN-gamma. Further study confirmed that addition of exogenous IFN-gamma to the co-culture of CD11b+Gr-1high cells and IFN-gamma KO T cells rescued the suppressive ability of CD11b+Gr-1high cells.

Gene expression analysis results indicated that in the absence of IFN-gamma, CD11b+Gr-1high MDSC upregulated the expression of the anti-apoptotic molecule BCL2A1. In contrast, BCL2A1 expression was down regulated by IFN-gamma. In addition the presence of IFN-gamma decreased the survival capacity CD11b+Gr-1high cells.

In conclusion, here we show for the first time that IFN-gamma counter regulates the function of monocytic CD11b+Gr-1dull/int. and granulocytic CD11b+Gr-1high cells.

Key Words: Animal model, Myeloid derived suppressor cell.

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MICROVESICLES (EXOSOMES) DERIVED FROM AML BLASTS CARRY IMMUNOSUPPRESSIVE MOLECULES AND INTERFERE ON THE IMMUNE CELL FUNCTIONS

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Background: Immunosuppressive blast-derived microvesicles (MV) are present in sera of untreated patients with acute myelogenous leukemia (AML) but not sera of normal controls (NC). These MV down-regulate immune cell functions, promoting tumor progression. We tested the hypothesis that blast-derived MV directly interact with lymphocytes, contributing to effecter T cell and NK cell dysfunctions.

Methods: MV were isolated from sera of AML patients at diagnosis (n=25) and from supernatant of tumor cell lines or NC (n=16)by exclusion chromatography and ultra centrifugation and were tested for the presence of CD9, CD34, CD33, CD117, TGF-beta 1, FasL, CD39, CD73 and MAGE3 by flow cytometry and Western blotting. Functional assays were MV-mediated down-regulation of NKG2D in normal human NK cells or T-cell proliferation in response to anti-CD3/CD28 Ab stumilation. MV were labeled with PKH26 and co-incubated with NK or T cells + antibodies preventing their surface binding or internalization by lymphocytes.

Results: Only blast-derived MV in AML patients expressed TGF-beta 1, FasL, MICA/B, CD73 and MAGE3. These MV down-regulated expression of NKG2D and inhibited proliferation of T cells. Mechanisms involved in the induction of MV-mediated immune cell dysfunction were related to the presence and different levels of expression of MICA/B, TGF-beta 1 and CD73 on individual patients' MV. Antibodies specific for these molecules were able to restore normal immune functions. Similarly, binding/uptake of MV

by T or NK cells were associated with decreased functions in these cells and could be inhibited by agents preventing the MV-uptake.

Conclusions: Blast-derived MV present in sera of AML patients at diagnosis carry immune-modulating molecules and are responsible, in part, for aberrations of anti-tumor immune responses which contribute to poor outcome in the disease.

Key Words: Leukemia, NK cells, Tumor microenvironment.

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ROLE OF IL6/IL8-NF-KB AXIS IN THE IMMUNOSUPPRESSION AND RESISTANCE TO IMMUNOTHERAPY

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It is important to understand mechanisms of immunosuppression in cancer patients for improvement of immunotherapy. Cancer cells trigger various immunosuppressive cascades which cause multiple immunosuppressive mechanisms. We have previously found that activation of various signaling pathways in human cancer cells, including MAPK, STAT3, Wnt/b-catenin, and Snail, causes immunosuppression through multiple immunosuppressive cells and cytokines. In this study, we have analyzed the immunosuppressive mechanisms caused by activated NF-kB in human ovarian cancer cells, evaluated the role of this immunosuppression in immunotherapy, and tested whether systemic administration of specific inhibitors might reverse the immunosuppression in vivo using a murine model. NF-kB pathway was frequently activated in human ovarian cancer. IL6 and IL8 highly produced by such ovarian cancer cells were significantly reduced by treatment with NF-kB inhibitor, DHMEQ. Culture supernatants from the ovarian cancer cell lines impaired function of monocyte-derived DC for IL12 production and T cell stimulatory activity partly due to IL6, and also induced immunosuppressive macrophages from human monocytes in vitro. These immunosuppressive effects of human ovarian cancer were reduced by DHMEQ treatment. When the human ovarian cancer cell line was implanted in nude mice, T cell stimulatory function of murine splenic DC was impaired, and CD11b+ Gr-1+ arginase+ murine MDSC were increased in spleen and tumor. Systemic administration of DHMEQ reversed the in vivo immunosuppression partly due to decrease of IL6 production. Transfer of murine naïve T cells into the nude mice bearing human ovarian cancer caused T cell dependent tumor regression, and combined administration of DHMEQ enhanced the T cell mediated anti-tumor effects. Increase of IL-6, IL-8 and arginase was frequently observed in plasma of patients with ovarian cancer. There was a positive correlation between IL-8 and arginase. We found that high IL6 and IL8 in plasma were correlated with less immune-induction and poor prognosis in cancer patients who received various cancer vaccines. These results indicate that IL6/IL8 / arginase - NF-kB axis is involved in immunosuppressive mechanisms of human cancers, and is associated with resistance to immunotherapy. Therefore, blockade of this axis may be useful for augmentation of anti-tumor effects of current immunotherapy.

Key Words: Cytokine, Targeted therapeutics, Tumor microenvironment.

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RE-PROGRAMMING OF TUMOR ASSOCIATED T-REGULATORY CELLS FOR IMPROVED CANCER IMMUNOTHERAPY

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The presence of T-regulatory cells (Treg) in the tumor microenvironment plays a significant role in the suppression of anti-tumor immunity and blocks many therapeutic benefits. In our previous studies, we have shown that IL-12 treatment can restore cytotoxic function to tumor-associated CD8+ T effector/memory cells and induce the apoptotic death of Treg via the FAS-FASL pathway. Further studies demonstrated that effector activation was transient, as the tumors were re-infiltrated by Treg within 5 to 7 days of treatment marking the end of tumor-specific CD8+ cytotoxic T-lymphocyte (CTL) activity. Furthermore, in repeated treatment regimens with IL-12, Treg rebound becomes a major issue as attempts to boost anti-tumor immunity resulted in intensification of the Treg rebound. These findings suggested that we revise our strategies in order to overcome counter-regulation. It was recently demonstrated that generation of pathogenic Th17 cells from naïve CD4+ T cell in the absence of TGF-β signaling is possible. Preliminary studies revealed a significant degree of plasticity in Treg isolated from tumor-draining lymph nodes of tumor-bearing mice. Specifically tumor-conditioned CD4+CD25+ Foxp3+ cells converted to a Th17(23) phenotype upon exposure to IL-1β, IL-6, IL-23 and IL-21. We also demonstrated that those cells could further convert a to TH1 phenotype in the presence of IL-12 and IL-2. Treg cell reprogramming could also be achieved using CD4+CD25+ Foxp3+ cells isolated from the TDLN of IL-12 treated mice. We are currently testing whether phenotypically reprogrammed Treg cells can contribute to antitumor immunity.

Key Words: Combination immunotherapy, Targeted therapeutics, Treg cells.

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IDENTIFICATION OF A PARTICULAR SUBSET OF MDSC WHICH HAS MULTIPLE EFFECTOR ROLES IN TUMORIGENESIS

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Myeloid Derived Suppressor cells (MDSC) make up an abnormally large portion of tumor infiltrating cells in patients and have been found to suppress anti-tumor immune responses by various mechanisms. We have recently identified a particular subset of MDSC; Ni-

tric Oxide (NO)-producing cells which play a direct effector role in attenuating CD8 T-cell cytotoxicity as well as indirect role in promoting tumor dependent angiogenesis, invasion and ultimately metastasis. After a detailed characterization of their phenotype and function, it was observed that they have a similar phenotype to Tumor-associated Macrophage (TAM) and they blocked the survival and proliferation of activated tumor-resident CD8+ T-effector/memory cells (Tem) to a great extent. They were resistant to the differentiation into mature macrophage or dendritic cell (DC) phenotype both in vivo and in vitro. We also demonstrated that blocking of iNOS activity with N-nitro-l-arginine methyl ester (L-NAME), dramatically enhanced tumor suppression revealing the inhibitory effect of NO on antitumor immunity. Another yet undefined role of MDSC is their contribution to angiogenesis and vasculogenesis. In vitro co-culture assays revealed that they contribute to the process of forming some structural units which might be the indication of sprouting blood-vessel layers. We are currently testing how this particular subset can contribute to tumor angiogenesis and vasculogenesis.

Key Words: Antiangiogenesis, Myeloid derived suppressor cell, Tumor microenvironment.

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INFLAMMATION-INDUCED IMMUNOLOGICAL SOIL AND PREVENTION OF BREAST CANCER BRAIN METASTASIS

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As therapies for systemic cancer improve and patients survive longer, the risk of brain metastases increases, yet we lack predictors of and effective therapies for brain metastases. Brain metastases of cancers, therefore, are major obstacles that must be overcome before cancers can be cured by any means. To study whether brain metastasis can be mediated by primary tumor-induced immunological pre-conditioning in the brain, we conducted the following studies using Balb/c mice and syngeneic 4T1 mammary adenocarcinoma cells. Mice bearing 4T1 tumors in the mammary pad present with accumulation of CD11b+Gr1+ myeloid cells, which are likely to be myeloid-derived suppressor cells (MDSCs), in the brains prior to any detectable tumor cell metastasis. We have also demonstrated that S100A8/A9, serum amyloid A (SAA)3 and CCL2, but not other common inflammatory cytokines, are up-regulated in the brain prior to metastatic spread of 4T1 cells. On the other hand, neither accumulations of MDSCs, nor up-regulation of S100A8/A9 and SAA3 is detected in the brains of mice bearing JC breast cancer cells which are not metastatic. Systemic treatment of 4T1-bearing mice with cyclooxygenase-2 (COX-2) inhibitor, celecoxib, reduces both CD11b+Gr1+ myeloid cell accumulation as well as expression levels of S100A8/A9, SAA3 and CCL2 in the pre-metastatic brains of 4T1 bearing mice. Furthermore, celecoxib treatment starting on Day 2 following the 4T1 cell inoculation in the mammary pad significantly inhibits brain metastasis of 4T1 cells detected on Day 30. Systemic treatment with anti-CCL2 (C1142) or anti-Gr1 (RB6-8C5) monoclonal antibodies (mAb) also reduces CD11b+Gr1+ myeloid cell accumulation as well as expression levels of S100A8/A9 and SAA3 in the pre-metastatic brains. Our results strongly suggest, for the first time, that tumor-derived inflammatory responses, including the induction of CCL2, may be responsible for priming the "pre-metastatic soil" in the brain, thereby promote metastasis. Celecoxib, anti-CCL2 or anti-Gr1 mAb treatment may be used to prevent the formation of pre-metastatic immunological soil. In particular, celecoxib may be useful for the prevention of brain metastasis in patients with breast cancer. Further understanding of the mechanisms underlying the immunological soil will allow us to develop more effective strategies to prevent brain metastasis of breast cancer.

Key Words: brain metastasis, breast cancer, myeloid-derived suppressor cells (MDSCs).

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PHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD AND ASCITES FLUID IN OVARIAN CANCER PATIENTS

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Ovarian cancer is a highly treatable disease if detected early yet suffers from a high mortality rate due to the often late stage of initial presentation. Our lab is interested in the classification of ovarian cancer patients based on peripheral immunity. We believe this classification will enhance immune based approaches to the disease, improve our understanding of how conventional treatments affect immunity and monitor the effects of novel immune therapies on treatment. As such, we measured the immunological profile of over 40 phenotypes in newly diagnosed or recently relapsed ovarian cancer patients by flow cytometry of peripheral blood and in ascites fluid. At the time of analysis all patients were off of all therapy for more than four weeks. We have previously phenotyped patients in glioblastoma and non-Hodgkin lymphoma and identified specific immune deficiencies including CD4 lymphopenia and loss of HLA-DR expression on CD14+ monocytes. In 13 ovarian cancer patients, we observed similar profiles including CD4 lymphopenia and loss of class II (HLA-DR) expression on CD14+ monocytes when compared to age and gender matched healthy volunteers ($p<0.05$). Additionally, ovarian cancer patients had reduced expression of HLA-DR on B cells and increased levels of platelets ($p<0.05$). In contrast, there were no changes seen in other immune suppressive phenotypes such as regulatory T cells (CD4+CD25+CD127low) or lineage-HLA-DR-CD33+ myeloid derived suppressor cells. In a limited sample of patients we also obtained corresponding ascites fluid and compared the immune profile in ascites with the patient's blood. In some patients we found large increases in the percentages of lymphocytes within the mononuclear infiltrating cell population and monocytes with high HLA-DR expression in ascites fluid com-

pared to blood samples. These findings show that some ovarian cancer patients present with a profound systemic immune suppressive profile while simultaneously presenting with an immune stimulatory profile in the peritoneum.

Key Words: Cellular immunity, Ovarian cancer.

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IMMUNE PROFILES OF PEDIATRIC CANCER PATIENTS

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The prognosis of advanced and relapsed sarcomas in pediatric patients is dismal with few treatment options. A prerequisite to immune therapies in this patient population is a comprehensive description of the deficits of immunity. To improve our understanding of immunity in pediatric sarcomas, we produced an immune profile representing >40 leukocyte phenotypes. Patients with osteosarcoma, Ewing sarcoma, or rhabdomyosarcoma were enrolled at time of diagnosis/relapse. Peripheral blood from 14 patients was collected before chemotherapy. Relapsed patients were off chemotherapy for a minimum of 4 weeks. Blood from 15 young adult healthy volunteers was collected as controls. The average age of patients was 15y (range 10-22y) and controls was 25y ($p<0.01$). In these patients, ratios of major populations of leukocytes were altered resulting in a greater frequency of granulocytes (67% v. 57%; $p<0.01$) and a reduced frequency of lymphocytes (21% v. 29%; $p<0.01$). By measuring the cells/uL within the lymphocyte pool, we determined that the deficiency was due mainly to the loss of CD4 T cells in sarcoma patients (727 cells/μL v. 1003 cells/μL; $p=0.06$). Analysis of the helper T cell pool suggested that the CD4 T cells were largely inhibitory as seen by the increased percentage of CTLA-4 expression (10.3% v. 2.8%; $p<0.01$). We found no difference in frequency of regulatory T cells (Treg, CD4+, CD25+, CD127low). Sarcoma patients had increased loss of HLA-DR expression on monocytes (MFI=337 v. 663; $p=0.04$) but not on B cells. These immune suppressive monocytes have been previously shown to inhibit antigen independent T cell proliferation and dendritic cell maturation. In contrast, there was no increase in a myeloid suppressor phenotype (Lin-, HLA-DR-, CD33+). Due to the significant age difference between these patients and controls, we also analyzed the data based on weight. The mean weight of these sarcoma patients was 59 kg (range 30-90 kg) and minimum weight for controls is 50 kg. We re-analyzed the data within patients grouped above and below 50 kg. We identified a single change, a reduction CD25 present on activated CD4 T cells in the smallest patients (17% v. 42%; $p<0.01$). These data demonstrate the potential for measuring the immune profile of patients, monitoring changes mediated by conventional or novel therapies and directing therapies to target the immune deficits in these patients.

Key Words: Tumor microenvironment.

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HYPOXIA DETERMINES CD137 FUNCTIONAL EXPRESSION ON TUMOR INFILTRATING T LYMPHOCYTES

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The response to hypoxia modulates the expression of multiple genes. The tumor microenvironment of transplanted and spontaneous mouse tumors is profoundly deprived of oxygen as confirmed by PET imaging. CD8 and CD4 tumor infiltrating T lympocytes of transplanted colon carcinomas, melanoma and spontaneous breast adenocarcinomas are CD137 positive, as opposed to their counterparts in tumor draining lymph nodes and spleen. Expression of CD137 on activated T lymphocytes is markedly enhanced by hypoxia and the prolyl hidroxilase inhibitor DMOG. Importantly, hypoxia does not up-regulate CD137 in inducible HIF-1alpha-/- T cells, and such HIF-1alpha deficient T cells remain CD137 negative even when becoming tumor infiltrating lymphocytes, in clear contrast with co-infiltrating HIF-1alpha- sufficient T cells. The fact that CD137 is selectively expressed on TILs was exploited to confine the effects of immunotherapy with agonist anti-CD137 mAb to the tumor tissue, thereby avoiding liver inflammation, while still permiting synergistic therapeutic effects with PD-L1/B7-H1 blockade.

Key Words: CD137 (4-1BB), microenvironment, Hypoxia.

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INTRACELLULAR EXPRESSION OF THE CO-INHIBITORY MOLECULE, B7-H4, IN NSCLC CELL LINES: IS IT REAL? WHAT DOES IT DO?

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The family of B7 ligands interact with the CD28 family of receptors on T cells to deliver either co-stimulatory or co-inhibitory signals. Shifting the balance of these signals during cancer immunotherapy likely impacts whether the developing anti-cancer immune response is tumor destructive or tolerized. B7-H4 is one of the B7 family members that has co-inhibitory activity and has been associated with poor immune responses and/or worse clinical outcome in melanoma, renal, ovarian, esophageal and gastric cancers, leading to the suggestion that it has a role in tumor immune evasion. Given our interest in NSCLC we evaluated a panel of 13 NSCLC cell lines for expression of B7-H4 and found only one cell line over expressed the gene. The other cell lines exhibited a similar expression level as RNA from normal lung. We next analyzed surface expression by flow cytometry and found 0/13 cell lines with detectable levels of the protein. Since a number of recent reports have identified intracellular expression of B7-H4, we stained for intracellular

levels of B7-H4 and found that 100% (10/10) of the cell lines were strongly positive for B7-H4 expression. Current efforts are focused on confirming expression findings and evaluating whether these cell lines secrete B7-H4.

Key Words: NSCLC, B7-H4 Co-inhibitory molecule, immune escape.

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TISSUE IMAGING VISUALIZES LYTIC DEFICITS IN TUMOR-INFILTRATING CD8 T LYMPHOCYTES IN SITU AND COMBINED WITH IN VITRO MODELS UNCOVERS A PIVOTAL ROLE OF THE TUMOR MICROENVIRONMENT IN CAUSING CELL DEVIATIONS RELATED TO TUMOR IMMUNE ESCAPE

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Human renal cell carcinoma is densely infiltrated with CD8 lymphocytes. Yet, tumors are not rejected suggesting that the tumor environment limits effector cell efficacy to control tumor growth.

To unravel deficits multiparameter fluorescence staining and confocal microscopy was performed determining the status of lymphocytes in direct physical contact with malignant cells and under the control of the local microenvironment. All CD8 lymphocytes in tumor tissue (CD8-TILs) were found equipped with lytic granules, yet more than 60% lacked perforin and granzyme B. A special image analysis, modeled on the process of lytic granule exocytosis, was applied to identify CD8-TILs with active tumor recognition. Synaptic lytic granule topology, a pattern which was associated with lytic function in in vitro models, was seen among perforin-positive but not perforin-negative CD8-TILs.

Although some TILs appeared actively engaged in tumor recognition there was no evidence that any CD8 cell was stimulated to produce IFNγ. Compared to control tissues with histopathologically verified lytic tissue destruction, RCC had significantly more perforin-negative and fewer functionally active CD8 T cells revealing a shift towards T cells with poor functional quality in RCC. Assessing the perforin status of CD8-TILs in relation to their spatial distribution within the tumor revealed a pivotal role of the tumor microenvironment: Most CD8-TILs that had extravasated into the tumor parenchyma were perforin-negative while those still residing in the tumor vasculature were largely perforin-positive. Thus, CD8 cells appear to arrive at the tumor site functionally proficient and become compromised within the tumor environment.

Ex vivo analyses identified TCR signaling alterations in CD8-TILs compared to CD8 T cells of non-tumor kidney which were associated with failure to degranulate. These deviations were reversible concomitantly with gain in perforin and function.

Application of in vitro models, which mimic conditions of solid tumors, identified tumor lactic acidosis as one potent factor abrogating TCR-stimulated IFNγ production by inhibition of p38 and JNK/c-Jun activation. Unidentified tumor cell-secreted factor(s) appear to cause loss of perforin and lytic function.

The results reveal perforin paucity and inhibition of CD8-TIL function imparted by the tumor environment as important mechanisms of immune escape in RCC. Identified alterations indicate options to modulate the tumor environment allowing maintenance of CD8-TIL function which could enhance the efficacy of immunotherapy.

Key Words: Tumor milieu, cytotoxic lymphocytes, functional deficits.

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POSITIVE FEEDBACK BETWEEN PGE2 AND COX2 DIVERTS DC DIFFERENTIATION INTO MDSCS AND SUSTAINS THEIR IMMUNOSUPPRESSIVE FUNCTIONS AND CXCL12/CXCR4-DEPENDENT TUMOR HOMING

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Myeloid-derived suppressor cells (MDSCs) are essential players in cancer-associated immune dysfunction. We report that the differentiation, functional stability, and homing properties of human monocytic MDSCs are driven by one ovarian cancer-associated inflammatory mediator, prostaglandin E2 (PGE2), and depend on the establishment of a positive feedback between PGE2 and the key regulator of PGE2 synthesis, COX-2. PGE2 abrogates the differentiation of CD1a+ DC from monocytes, redirecting it towards CD14+CD33+CD34+ cells expressing all MDSC-associated suppressive factors: IDO, arginase, NOS2 and IL-10, and inducing their CTL-suppressive functions. Moreover, PGE2 promotes the expression of endogenous COX2 and autocrine secretion of PGE2 in MDSCs, establishing a positive feedback loop that stabilizes the MDSC phenotype. Undisturbed COX2 activity and persistent production of PGE2 by MDSCs, isolated from cancer patients, was required for their continued expression of CXCR4 and responsiveness to CXCL12. Moreover, PGE2 also proved to be the key factor promoting the production of CXCL12 in cancer microenvironment, a chemokine exerting multiple tumor-promoting effects either directly by acting on cancer cells or indirectly by inducing angiogenesis and recruiting other immunosuppressive cells, i.e. Tregs and pDCs. In accordance with the physiological role of PGE2 in the development and functional stability of human cancer -associated MDSCs, we observed that the frequencies of CD11b+ CD33+ MDSCs in the ovarian cancer (OvCa) ascites closely correlate with the local production of PGE2 and expression of COX2, while the OvCa ascites environment can promote the induction of MDSCs from differentiating monocytes in a COX2-dependent manner. Importantly. disruption of COX2-PGE2 feedback in fully-developed MDSCs isolated from cancer patients, using COX2 inhibitors or inhibitors of EP2/EP4-dependent PGE2 signaling, eliminates the production of all other suppressive factors and terminates the CTL-suppressive function of MDSCs as well as eliminates both the expression of CXCR4 on MDSCs and the production of CXCL12,

highlighting the key role of PGE2 in the tumor-accumulation of immunosuppressive cells. The currently demonstrated key role of the positive COX2-PGE2 feedback loop in the differentiation and functional stability of MDSCs facilitates the targeting of MDSCs in cancer immunotherapy and facilitates the development of additional MDSC-targeting therapies.

Key Words: Cell trafficking, Myeloid derived suppressor cell, Ovarian cancer.

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CD4+CD39+FOXP3+ REGULATORY T CELLS HYDROLYZE ADENOSINE TRIPHOSPHATE AND MAINTAIN AN INTENSE CROSS-TALK WITH CD39+FOXP3NEG EX-TREG

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Background. We have shown that the ectonucleotidase CD39 is a surface marker for discrimination and isolation of human CD4+ regulatory T cells (Treg). Here, we evaluate CD4+CD39+FOXP3+ Treg functions as well as their interaction with CD4+CD39+FOXP3neg ex-Treg and conventional T cells (Tconv) in normal donors and patients with cancer.

Methods. Peripheral blood samples were obtained from normal donors (n=20) and patients with cancer (n=60). The frequency of Treg and ex-Treg in PBMC was monitored by multi-parameter flow cytometry. CD4+CD39+, CD4+CD39neg and CD4+CD73+ T cells were isolated using antibody-coated beads. Surface and intracellular markers and cytokine expression after stimulation were assessed by 10-color flow cytometry. In Treg and T cells, CD39 and CD73 expression was determined by realtime-PCR (mRNA) and western blot (protein). Adenosine production was measured by mass spectrometry. Cross-suppression was monitored in co-cultures of Treg and ex-Treg. Induction and expansion of Treg was evaluated in CD4+CD39+ and CD4+CD39neg T cells cultured in the presence of OKT3/anti-CD28 Ab-coated beads, IL-2 ± TGF-β.

Results. Cross-talk between the two CD4+CD39+ subsets has led to suppression of Th1 cytokine expression in CD39+FOXP3neg ex-Treg and down-regulation of FOXP3 expression in CD39+FOXP3+ Treg. Induction of CD39+FOXP3+ was TGF-β dependent, while that of CD39+FOXP3neg T cells was not. In co-cultures with IL-2, both subsets expanded equally well, and up-regulated CD39 and FOXP3 independently of TGF-β. Only CD39+FOXP3neg ex-Treg were PD1+, and their cytokine expression was enhanced in the presence of anti-PD1 Ab. CD4+CD39+ T cells hydrolyzed ATP to AMP, which was then hydrolyzed to adenosine only in the presence of CD4+CD73+ T cells. Both subsets of CD4+CD39+ T cells were equally increased in HNSCC patients, and their frequencies were significantly correlated ($p < 0.001$).

Conclusion. ATP-hydrolyzing Treg and non-suppressive ex-Treg subsets co-exist in the peripheral blood of NC and cancer patients. These subsets maintain an intense cross-talk, regulating their respective functions and controlling suppression.

Key Words: Chemotherapy, Cytokine, Treg cells.

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IMMUNOSUPPRESSION IN TRANSGENIC MOUSE MODEL OF SPONTANEOUS SKIN MELANOMA AND ITS NEUTRALIZATION

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Melanoma is known for its poor response to current immunotherapies. Insufficient anti-tumor reactivity could be due to the formation of a chronic inflammation represented by infiltrating leukocytes and soluble mediators, which lead to cancer progression. Here, we used the ret transgenic spontaneous murine melanoma model that mimics human melanoma showing in skin tumors and metastatic lymph nodes increased levels of inflammatory factors such as IL-1beta, GM-CSF, and IFN-gamma, which correlate with tumor growth. Moreover, Gr1+CD11b+ myeloid derived suppressor cells (MDSC) known to inhibit tumor reactive T cells were enriched in melanoma lesions and lymphatic organs during tumor progression. MDSC infiltration was associated with a strong TCR zeta-chain down-regulation. Co-culturing normal splenocytes with tumor-derived MDSC induced a decreased T cell proliferation and zeta-chain expression, verifying the MDSC immunosuppressive function. These results suggest that tumor inflammatory microenvironment supports MDSC recruitment and immunosuppressive activity. Indeed, upon manipulation of the tumor microenvironment in melanoma bearing mice with the phosphodiesterase-5 inhibitor sildenafil, we observed reduced amounts of inflammatory mediators (IL-1beta, VEGF, and GM-CSF) and immunosuppressive factors (nitric oxide and arginase-1) in association with decreased MDSC levels and immunosuppressive function. These led to the restoration of zeta-chain expression in T cells and to a significant increased survival of treated mice.

Other treatments (like chemomodulation with very low, non-cytotoxic and non-cytostatic doses of paclitaxel, or application of the proton pump inhibitor omeprazol) also led to a substantial retardation of melanoma progression associated with an inhibition of chronic inflammatory mediator production in melanoma lesions and with a reduction of MDSC immunosuppressive activity. Our data suggest that inhibitors of the immunosuppressive tumor microenvironment induced by chronic inflammation should be applied in conjunction with melanoma immunotherapies to increase their efficacy.

Key Words: melanoma immunotherapy, immunosuppression.

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LISTERIA MONOCYTOGENES-BASED CANCER IMMUNOTHERAPIES REDUCE IMMUNOSUPPRESSION IN THE TUMOR MICROENVIRONMENT

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Previously we have shown that the expression of the Tumor Associated Antigen (TAA) using recombinant Listeria monocytogenes (Lm) causes the induction of specific T cell responses which are required for tumor regression. However, to maximize therapeutic responses it is necessary to not only generate T cell responses but also to effect intra-tumoral immune suppression. Using two different tumor models, TRAMPC-1 expressing human PSA (TPSA23) and NT-2 mammary carcinoma, we have shown that immunization with live attenuated Lm-based immunotherapies results in decreases in the frequency of CD4+CD25+FoxP3+ regulatory T cells (Tregs) in the tumor microenvironment. Reduction in regulatory T cells was observed in response to all Lm-based immunotherapies, in the tumor microenvironment but not in other peripheral immune organs such as spleen or draining lymph nodes. Recently we observed a similar localized tumor effect in Myeloid Derived Suppressor Cells (MDSC). Interestingly, beyond decreases in cell counts, MDSC in the tumor microenvironment exhibit reduced ability to suppress T cell function. The functional decrease in the ability of MDSC to suppress T cells is observed locally in tumors but not in other peripheral immune organs such as spleens. Currently, we are conducting studies to delineate the mechanism for this alteration in MDSC function. The outcome of these studies will impact future clinical studies with Lm-based immunotherapies and/or other combination therapies which may result in improved outcomes in the treatment of cancer.

Key Words: Myeloid derived suppressor cell, Treg cells, Tumor microenvironment.

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THE IMMUNOSUPPRESSIVE ROLE OF WNT/BETA-CATENIN SIGNALS IN MELANOMA CANCER MICROENVIRONMENTS

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Cancer-induced immunosuppression is the major problem reducing anti-tumor effects of immunotherapies, but its molecular mechanism has not been well understood. Wnt/β-catenin pathway is frequently activated in various cancers including human malignant melanoma (MM) and contributes to their malignant phenotypes. In this study, we demonstrated that the Wnt/β-catenin signal had immunosuppressive roles through production of IL10 and Histatin-1 (HTN-1) in human MM using various methods including DNA chip analysis, RT-PCR, IHC, reporter assays, and gene knockdown or overexpression experiments. An immunosuppressive cytokine IL10 and HTN-1, an antimicrobial peptide specifically expressed in parotid saliva, were found to be ectopically expressed in human MM cell lines and tissues by activation of the Wnt/β-catenin signal pathway. Culture supernatants (CS) from β-catenin-accumulated MM had activities to induce possible tolerogenic DC producing low IL12, and high IL10, and its immunosuppressive activities were reduced by knocking-down of β-catenin in MM cells. A HTN-1 peptide or IL10 into the culture generating dendritic cell (DC) from human monocytes induced tolerogenic DC, indicating that HTN-1 and IL10 were involved in the immunosuppression induced by MM with the activated Wnt/β-catenin signal. The function of murine splenic DC obtained from nude mice implanted with human mutant β-catenin-overexpressed MM cells which produced IL10 and HTN-1 were suppressed, compared with those from the mice with control MM, and this suppression was restored by the systemic administration of a β-catenin inhibitor, showing in vivo suppression of DC by the activated Wnt/β-catenin signal in human MM. The number and the function of tumor-infiltrating DC from nude mice implanted with HTN-1-overexpressed human MM were also decreased. Interestingly, β-catenin-overexpressed MM was found to be more resistant to CTL lysis and to inhibit IFN-γ production by MM specific activated CTL in an IL10 or HTN-1 independent manner. These results indicate that Wnt/β-catenin signal may be involved in the immunosuppression by human MM in both induction and effector phases of anti-tumor immune responses partly due to increased production HTN-1, of which immunosuppressive functions were newly identified in this study, and IL10 and may be an attractive target for restoring immunocompetence of patients with Wnt/β-catenin-activated MM.

Key Words: Melanoma, Targeted therapeutics, Tumor microenvironment.

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HUMAN CCR4+CCR6+ TH17 CELLS SUPPRESS AUTOLOGOUS CD8+ T CELL RESPONSES

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Th17 cells are a novel CD4+ T helper cell subset that plays an important role in the pathogenesis of autoimmune diseases. However, their role in cancer patients remains unclear and controversial. We observed high amounts of IL-17 and IL-23 in tumor supernatants from hepatocellular carcinoma (HCC) patients. HCC patients also showed higher frequency of CD4+IL-17+cells in the peripheral blood than healthy donors and non-cancer patients with hepatitis C virus (HCV) infection. Therefore we decided to examine the function of CD4+IL-17+ cells in more detail and amplified these cells in vitro from naïve CD4+ T cells. CD4+ T cells were isolated from peripheral blood and expanded in the presence of IL-1-beta, IL-6, IL-23 and anti-IL-4 and anti-IFN-gamma. Two subpopulations

were detected in the in vitro culture. CCR4+CCR6+ Th17 cells express high amount of IL-17 and low levels of IFN-gamma. Co-culture of CCR4+CCR6+ Th17 with CD8+ T cells revealed that CCR4+CCR6+ Th17 cells suppressed the proliferation and IFN-gamma secretion of CD3/CD28 stimulated autologous CD8+ T cells in contrast to CCR4-CCR6+ CD4+ T cells, which were used as a control. In vitro generated CCR4+CCR6+ Th17 cells displayed a similar phenotype and function to freshly ex vivo isolated Th17 cells. Finally CCR4+CCR6+ Th17 cells also suppressed antigen-specific lysis and IFN-gamma production by antigen-specific CD8+ T cells. Suppression of CCR4+CCR6+ Th17 cells was not due to FoxP3+ regulatory T cell contamination and was cell-contact dependent. The possible suppressive mechanism needs to be further elucidated. Our study demonstrates a novel suppressive activity of the Th17 subset. Only CCR4+CCR6+ Th17 cells displayed this phenotype underlying the importance to examine the function of Th17 cell subsets. We believe that our findings will have important implications for the future design of effective immunotherapy protocols for patients with cancer.

Key Words: CD4+ T cells.

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DYSFUNCTIONAL TUMOR-INFILTRATING T CELLS EXPRESS THE ANERGY-ASSOCIATED MOLECULES LYMPHOCYTE-ACTIVATION GENE 3 (LAG3) AND CLASS-I-MHC RESTRICTED T CELL ASSOCIATED MOLECULES (CRTAM)

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Anergy is one of the mechanisms contributing to T cell dysfunction in the tumor microenvironment. However, the lack of a specific marker to define anergic T cells, along with the fact that anergy has generally been defined by a process (TCR ligation in the absence of costimulation) in concert with a subsequent dysfunctional state (the associated defective TCR/CD28-mediated activation), has limited the ability to clearly identify anergic cells. We have recently reported that the transcription factor early growth response factor 2 (Egr2) is a central regulator of T cell anergy, and deletion of Egr2 prevents anergy induction in vitro and in vivo. In an effort to map the complete Egr2 transcriptome of anergic T cells, we combined Egr2-driven gene expression profiling and ChIP-seq, and identified a set of cell surface molecules including Lag3 and Crtam, which were highly upregulated in anergic cells. Lag3 and Crtam have been implicated in regulating T cell activation, and we proposed that they might be useful markers to identify the anergic phenotype. To analyze the expression of Lag3 and Crtam in the context of tumor-induced T cell dysfunction, C57BL/6 mice were subcutaneously injected with SIY-expressing B16 melanoma, and tumor-infiltrating lymphocytes (TILs) were analyzed by flow cytometry. Crtam and Lag3 were upregulated in 40-60% TILs, on both CD4+ and CD8+ subsets, and their expression was largely overlapping. Interestingly, these cells represented a subpopulation of PD-1+ cells. Functionally, upon restimulation with anti-CD3/CD28 mAbs in vitro, Lag3+Crtam+ T cells were defective in IL-2 production, whereas the remainder of the PD-1+ T cells were functionally intact. In contrast, IFN-γ production was mostly preserved, similar to the phenotype of T cells anergized in vitro. Additional analysis revealed, among the CD4+ T cells, a large fraction of the Lag3+Crtam+ TILs were Foxp3+, representing T regulatory cells (Tregs). Thus, upregulated expression of Lag3 and Crtam could indicate antigen-activated Tregs. The CD8+Lag3+Crtam+ cells were Foxp3-. Analysis of human CD8+ TILs also revealed constitutive expression of the anergy-associated transcription factor Egr2. Further characterization of the Lag3+Crtam+ TILs is ongoing to interrogate for other definitions of the anergic state, including expression of defined anergy-associated genes, defective TCR-induced ERK phosphorylation, and reversibility of dysfunction upon proliferation to homeostatic cytokines. In addition to Lag3 and Crtam serving as cell surface markers to identify anergic T cell, they might be useful therapeutic targets for immunotherapy aimed to reverse TIL dysfunction.

Key Words: Treg cells, Tumor infiltration lymphocytes, Tumor microenvironment.

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