Extended Abstracts for
the 45th International Symposium of
the Princess Takamatsu Cancer Research Fund

RECENT ADVANCES IN CANCER IMMUNOTHERAPY

November 18-20, 2014
Tokyo, Japan

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Printed in Japan
ISSN 2186-8603

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IMMUNE REGULATION FOR CANCER IMMUNOTHERAPY

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Cancer immunotherapy is now in a new era as illustrated by recent clinical results showing effectiveness of immune-checkpoint blockade and cell therapy with tumor-killing T cells. Therapeutic vaccination with tumor antigens has been widely explored in the clinic as an antigen-specific cancer immunotherapy since the molecular characterization of tumor antigens that are recognized by tumor-reactive antibodies and cytotoxic T-lymphocytes (CTLs) in cancer patients. One of the key issues for improving the efficacy of the current cancer immunotherapy is to control various immunosuppressive elements present in cancer patients. In particular, given that many tumor-associated antigens recognized by autologous tumor-reactive CTLs are antigenically normal or slightly modified self-constituents, tumor immunity is, in part, an autoimmunity. This means that the mechanisms for maintaining immunological self-tolerance may hinder effective tumor immunity in cancer patients.

Central and peripheral mechanisms of self-tolerance are indeed operating on T cells recognizing tumor-associated self or quasi-self antigens[1]. As a typical illustration of this, $CD8^+$ T cells recognizing Melan-A/MART-1, a self-antigen expressed by normal melanocytes and some melanoma cells, are subjected to negative selection in the thymus; and those having escaped thymic deletion are subjected to active suppression in the periphery by regulatory T (Treg) cells, rendered anergic, or stay dormant[2-6]. In addition, cancer cells or antigen-presenting cells (APCs) may attenuate effector activity of tumor-reactive T cells via co-inhibitory molecules (such as PD-1 and CTLA-4) expressed by effector T cells and via immuno-suppressive molecules (e.g., cytokines such as TGF-β, IL-10, and tryptophan metabolites) produced by cancer cells and stromal cells including

APCs. A current key issue is therefore to determine which mechanism(s) of peripheral self-tolerance or which aspect(s) of immune suppression can be practically manipulated to evoke and enhance effective tumor immunity.

Among key mechanisms of peripheral self-tolerance, recent studies have shown that Treg cells play an essential role in sustaining self-tolerance and immune homeostasis by suppressing a wide variety of physiological and pathological immune responses against self and nonself antigens, as well as quasi-self tumor antigens[7]. The most physiologically relevant Treg cell population is $CD25^+CD4^+$ Treg cells, which are naturally present in the immune system as ~10% of $CD4^+$ T cells, specifically expressing the transcription factor Foxp3 (forkhead box P3 transcription factor), and largely produced by the thymus as a functionally distinct population with a self-skewed TCR repertoire[7,8]. Evidence is now accumulating in humans and rodents that $Foxp3^+CD25^+CD4^+$ Treg cells dominantly infiltrate into tumor tissues, forming a sizable fraction of tumor-infiltrating lymphocytes, and apparently impeding immune responses to tumor cells[9]. Depletion of Treg cells is indeed able to evoke effective tumor immunity in otherwise non-responding animals without serious autoimmunity if the duration and the degree of Treg depletion is controlled[10]. As a key mechanism of Treg cell-mediated suppression, Treg cells appear to control APC functions, in particular, down-regulate their expression of the co-stimulatory molecules CD80 and CD86 to various extents, thereby driving responder T cells into distinct cell fates, depending on TCR affinity of the latter for tumor-associated or self-antigens[6,11]. For example, with low expression of CD80 and CD86 by APCs, high affinity T cells for a self- or tumor-associated antigen become deleted by apoptosis, intermediate ones rendered anergic, and low affinity ones kept dormant (ignorant)[6]. Since anergic or dormant $CD8^+$ T cells can be distinguished functionally and phenotypically (e..g., the former being phenotypically naïve but highly expressing the co-inhibitory molecule CTLA-4), both populations specific for a particular self/tumor antigen (e.g., Melan-A/MART-1) have been shown to be present in the peripheral blood of healthy individuals and also tumor tissues where Treg cells are abundant (6, and our unpublished data). In tumor immunology setting, it is dormant naïve type T cells specific for tumor antigens that are able to become activated and expand upon tumor antigen vaccination, hence capable of tumor killing, because anergic type T cells are profoundly hypo-responsive and prone to die by apoptosis upon stimulation[6].

Thus, a variety of T-cell populations are present in tumor tissues and presumably in regional lymphoid tissues; e.g., Treg cells, naïve T cells, anergic T cells, exhausted T cells, and activated T cells. Each can be phenotypically distinguished in tumor tissues by the expression of specific cell surface molecules (Figure 1). For example, tumor-infiltrating Treg cells, in particular terminally differentiated and most suppressive effector Treg cells,

are generally high in the expression of CTLA-4, PD-1, and the chemokine receptor CCR4. Anergic $CD8^+$ T cells are much higher in CTLA-4 expression than Treg cells or activated T cells[6]. PD-1 is expressed by exhausted T cells, some anergic $CD8^+$ T cells, and early activated $CD8^+$ T cells in addition to tumor-infiltrating Treg cells[12]. It is therefore likely that agonistic, antagonistic, blocking, or cell-depleting antibodies specific for these molecules may exert different effects on anti-tumor immune responses as a whole. For example, cell-depleting anti-CCR4 depletes effector Treg cells; an anti-CTLA-4 mAb has a Treg-depleting effect in tumor tissues, but not in the circulation, in mice[13,14]. Anti-PD-1 appears to be blocking and able to re-juvenile exhausted $CD8^+$ T cells[12]. Whether anti-PD-1 has a Treg-depleting effect in tumor tissues remains to be determined. It is certainly envisaged that proper combinations of these antibodies specific for different T-cell surface molecules and with distinct T-cell function-modulatory effects will synergistically enhance anti-tumor immunity.

In addition to T-cell components discussed above, tumor microenvironments are composed of various cellular elements including cancer cells, cancer stromal cells, and vascular cells, which may suppress tumor immunity by various ways. It is hoped that coordinate targeting of these T and non-T cellular elements and their products, together with antigenic stimulation such as tumor antigen vaccination, will make cancer immunotherapy a major and key modality of cancer treatment in near future.

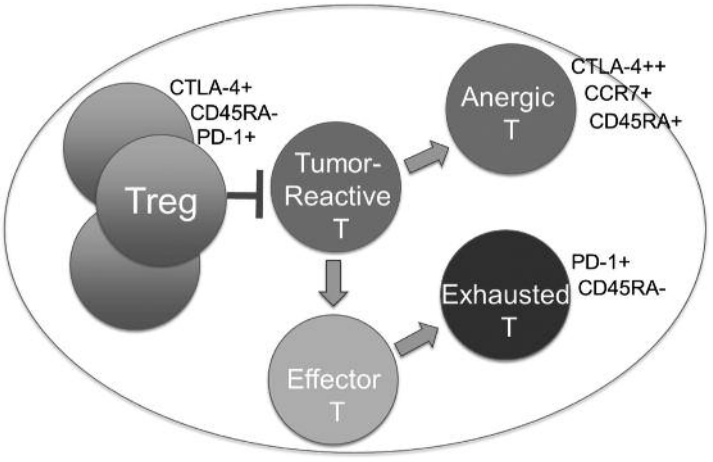

Figure 1 T cells in tumor tissues

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Specialty and Present Interest:
Immune Regulation, Autoimmune Disease, Tumor Immunity

TRANSLATING CANCER IMMUNOEDITING PRINCIPLES TO CANCER IMMUNOTHERAPY

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Cancer Immunoediting is the process by which the immune system controls and shapes cancer. We originally envisaged and subsequently showed that, in its most complex form, cancer immunoediting occurs in three phases: Elimination (also known as cancer immunosurveillance, the host protective phase of the process), Equilibrium (the phase in which tumor cells that survive immune elimination remain under immunologic growth control resulting in a state of functional tumor dormancy) and Escape (the phase where clinically apparent tumors emerge because immune sculpting of the tumor cells has produced variants that display either reduced immunogenicity or enhanced immunosuppressive activity)[1-3] (Figure 1). Strong experimental data have been obtained using mouse cancer models to demonstrate the existence of each phase of the cancer immunoediting process and compelling clinical data suggest that a similar process also occurs during the evolution of certain types of human cancer[4]. Our efforts now focus on elucidating the molecular and cellular mechanisms that underlie each phase of cancer immunoediting, identifying the critical checkpoints that regulate progression from one phase of the process to the next and defining the targets and mechanisms underlying the cancer immunoediting process.

In 2012, we reported one of the first experimental uses of cancer exome sequencing and epitope prediction algorithms to show that (a) mutant proteins in highly immunogenic tumor cells derived from methylcholanthrene treated immunodeficient mice represent immunodominant, tumor-specific mutant antigens for $CD8^+$ T cells; (b) these highly immunogenic tumor-specific mutant antigens represent major targets of cancer immunoediting and (c) that T cell dependent immunoselection for tumor cells that lack

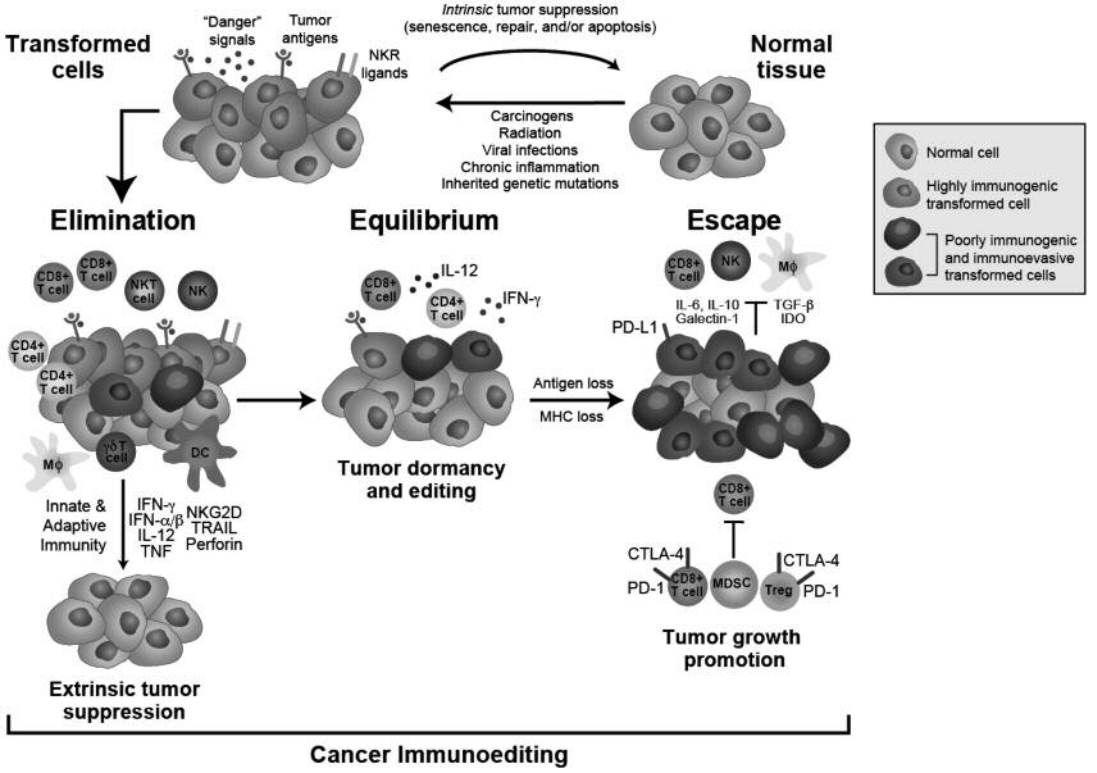

Figure 1 Cancer Immunoediting

expression of strong tumor specific mutant antigens is a major mechanism by which immunoediting occurs[5].

More recently, we asked whether our genomics/bioinformatics based approach could identify the antigens in progressively growing T3 sarcomas that render them susceptible to checkpoint blockade immunotherapy[6]. T cell lines generated from anti-PD-1 treated mice that had rejected T3 progressor sarcoma cells displayed restriction to H-2K^b but not to H-2D^b, suggesting that anti-PD-1 promotes T cell responses to only a limited number of antigens. We then identified expressed non-synonymous mutations in T3 tumor cells using cDNA Capture Sequencing and generated a prioritized list of potential H-2K^b binding epitopes based on predicted binding affinity. Filters were then applied to this list to take into account the likelihood that the mutant protein would undergo proteasomal processing and whether the predicted mutant neo-epitope displayed an equal or enhanced ability to bind to MHC class I compared to the corresponding wild type sequence while also deprioritizing predicted epitopes from hypothetical proteins. Based on these criteria, two unequivocal "best candidates" were predicted —mutant forms of Laminin α subunit 4 (mLama4) and a glucosyltransferase (mAlg8) (Figure 2). To test the validity of these

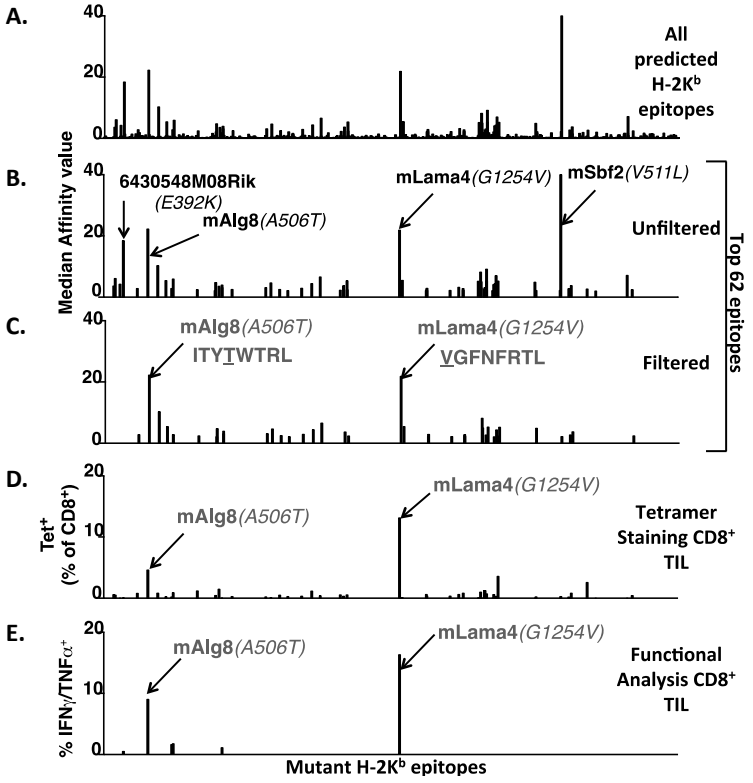

Figure 2 Mutations in Lama4 and Alg8 form top predicted d42m1-T3 epitopes
A. Potential H-2K^b binding epitopes predicted by *in silico* analysis of all missense mutations in the T3 sarcoma. **B**. Median affinity values for the top 62 predicted H-2K^b epitopes. **C.** Median affinity values of H-2K^b epitopes after filtering. **D.** Screening for specificities of CD8$^+$ TILs from αPD-1 treated, T3 sarcoma bearing mice using H-2K^b tetramers loaded with top 62 H-2K^b epitopes. **E.** IFN-γ and TNF-α induction in CD8$^+$ TILs from αPD-1 treated, T3 sarcoma bearing mice following culture with irradiated splenocytes pulsed with the top 62 H-2K^b peptides. Data are presented as per cent CD8$^+$ TILs expressing IFN-γ, TNF-α or for both. Data are representative of two independent experiments.

predictions, we synthesized 8 amino acid peptides representing the top 62 predicted H-2K^b epitopes and assessed whether they were recognized by CD8$^+$ TILs isolated from T3 tumors when presented by H-2K^b. Only the top two predicted epitopes—mLama4 and mAlg8—scored positive when either used in labelled H-2K^b tetramers to stain CD8$^+$ TILs from T3 tumors (as assessed by flow cytometry) or when the CD8$^+$ TILS from T3 tumors were incubated with irradiated splenocytes that had been pulsed with each of the predicted H-2K^b epitopes and T cells subsequently analyzed by flow cytometry for intracellular IFNγ and TNFα production. These findings were further validated by showing that: (a) T3 tumor-specific CD8$^+$ T cell lines generated from mice that had rejected T3 tumors following treatment with anti-PD-1 recognized the same two epitopes; (b) the mLama4 and mAlg8

epitopes were detected by mass spectrometry within the pool of peptides bound to H-2K^b expressed on T3 tumor cells; (c) CD8$^+$ T cells expressing TCRs for either mLama4 or mAlg8 accumulated over time in T3 tumors in anti-PD-1-treated, tumor-bearing mice, reaching maximal numbers just prior to tumor rejection; (d) vaccination of naïve WT mice with mutant but not WT forms of Lama4 or Alg8 induced strong CD8$^+$ T cell responses; and (e) naïve mice vaccinated prophylactically against mLama4 plus mAlg8 in the presence of poly I:C controlled outgrowth of T3 tumors. Most importantly, the mLama4 plus mAlg8 vaccine also caused the elimination of *established* T3 tumors, in a manner that was very similar to T3 tumor bearing mice treated with either anti-PD-1 and/or anti-CTLA-4 (Figure 3). These findings reveal that the combination of genomics, bioinformatics and immunologic approaches can indeed identify key tumor-specific mutant proteins that can function therapeutically as tumor-specific mutant rejection antigens and thus provide a basis for their use in the development of personalized therapeutic cancer vaccines.

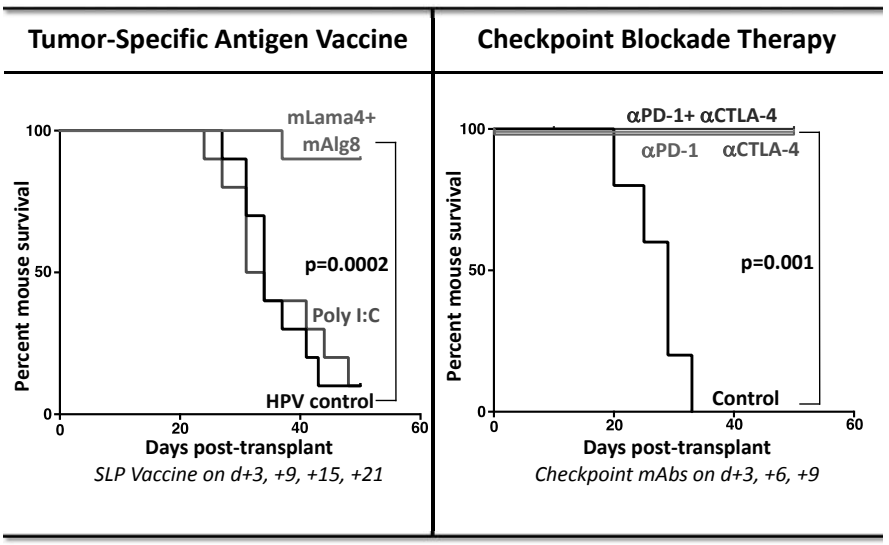

Figure 3 Combined mLama4 plus mAlg8 vaccine is nearly as effective as checkpoint blockade immunotherapy.
Groups of mice were treated with 1 x 10^6 T3 sarcoma cells and on day +3, when tumors had become established, groups of mice were treated either with: **Left Panel:** a synthetic long peptide (SLP) vaccine consisting of mLama4 plus mAlg8 plus 100μg poly I:C on days +3, +9, +15 and +21 (controls included treatment with an irrelevant SLP plus poly I:C vaccine or poly I:C alone) or **Right Panel:** 200μg each anti-PD-1, or anti-CTLA-4 or the combination of anti-PD-1 plus anti-CTLA-4 administered on days +3, +6 and +9. Tumor growth was measured and plotted as the mean of two perpendicular diameters. Mice were euthanized when tumors reached 20mm in diameter, which was the point used to generate the Kaplan-Meier survival curves.

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Specialty and Present Interest:
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CANCER AS A DISEASE OF THE METAORGANISM

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Commensal microorganisms colonize barrier surfaces of all multicellular organisms, including those of humans. For more than 500 million years commensal microorganisms and their hosts have coevolved and adapted to each other[1]. As a result, the commensal microbiota affects many immune and non-immune functions of their hosts, and de facto the two together comprise one metaorganism[1].

The commensal microbiota communicates with the host via biologically active molecules. The interplay between the host immune system and the microbiota prevents tissue-damaging inflammatory responses to the commensals and controls the growth of indigenous pathobionts while it sets the stage for immune responses against pathogenic infections[2-4]. This homeostatic immune regulation may be disrupted by changes in the microbial community that alter the symbiotic relationship with the microbiota and the resultant microbial imbalance is commonly referred to as dysbiosis[5]. Many regulatory mechanisms involved in these local interactions have been elucidated[6]. In addition to local immunity, the commensal microbiota regulates systemic inflammation, innate resistance and adaptive immunity affecting both resistance to infection and autoimmunity[7-12]. Recently, it has been reported that microbial imbalance may play a critical role in the development of multiple diseases, such as cancer, autoimmune conditions and increased susceptibility to infection[2].

The commensal microbiota not only may affect the development, progression and immune evasion of cancer but it has also important effects on the response to cancer immune- and chemo-therapy[3-5]. Myeloid cells are a major component of the tumor microenvironment where they play a dual role inducing anti-tumor immune responses but

mostly promoting immune evasion, tumor progression and metastases formation[6]. Thus, strategies aiming at reprogramming the tumor microenvironment represent a promising immunotherapy approach. Myeloid cells respond to environmental factors including signals derived from commensal microbes that modulate their function and reactivity thus impacting the response to cancer therapy[5].

The gut microbiota influences the response to cancer immunotherapy and chemotherapy by affecting the differentiation and functions of myeloid cells in the tumor microenvironment (Figure 1). Intratumoral injection of CpG-oligodeoxynucleotide (CpG-ODN) combined with antibody neutralization of IL10 signaling is a very effective treatment of large transplanted subcutaneous tumors in conventional mice but it is largely ineffective in GF or antibiotics-treated mice[5]. Within hours following CpG-ODN and anti-IL10R treatment tumors undergo an extensive hemorrhagic necrosis that is dependent on TNF and nitric oxide (NO) production by tumor-infiltrating myeloid cells[7]. DCs are then activated, and they migrate to the draining lymph nodes where they induce a CD8 T cell-mediated tumor-specific response required for tumor eradication. In antibiotics-treated or

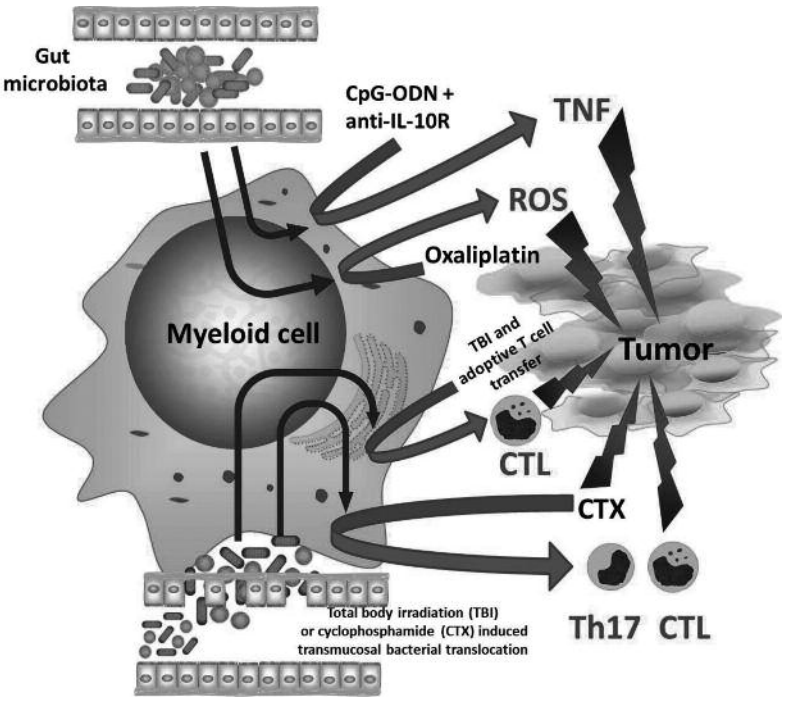

Figure 1 The gut microbiota influences the response to cancer immunotherapy and chemotherapy by affecting the differentiation and functions of myeloid cells in the tumor microenvironment with different mechanisms involved in the different types of immune- or chemo-therapy. For details, please see text.

GF mice, tumor-infiltrating myeloid-derived cells fail to produce inflammatory cytokines, including TNF and IL12, in response to CpG-ODN[5]. Oral gavage of antibiotics-treated mice with LPS partially rescued the deficient response to CpG-ODN[5]. Tumors from antibiotics-treated mice contained a lower number of monocyte-derived $Ly6C^{+}$ MHC-class II^{+} macrophage-like cells while the number of $Ly6C^{high}$ MHC class II^{-} inflammatory monocytes was equivalent to that of control mice without antibiotics treatment[5]. Tumor-associated myeloid-cell subsets have been shown to be mostly derived from circulating inflammatory monocytes that differentiate *in situ*[8-10]. Although inflammatory monocytes appear to infiltrate the tumors in equivalent number, their differentiation after reaching the tumor microenvironment is altered in the absence of gut microbiota and this may affect their response to CpG-ODN[5]. The fecal microbiota composition in mice showing high and low TNF responses to CpG-ODN appeared distinct. In particular, the abundance of several individual $Gram^{+}$ and $Gram^{-}$ bacterial spp positively correlated with the CpG-ODN response, whereas the presence of commensal *Lactobacillus* spp decreased the response[5]. *In vivo* association experiments confirmed that the $Gram^{-}$ *Alistipes shaii* enhances the CpG-ODN response while *L. fermentum* attenuates it[5].

The effect of the microbiota on chemotherapy was analyzed utilizing platinum compounds (e.g. oxaliplatin, cisplatin)[5]. These compounds mediate genotoxicity by forming platinum DNA-adducts followed by formation of intrastrand cross-links that inhibit protein synthesis and proliferation, and induce apoptosis in part downstream of the ataxia telangiectasia and rad3-related (ATR) kinase recruitment to DNA damage and p53 activation[11]. The therapeutic effect of oxaliplatin and cisplatin on mouse sterile subcutaneous transplanted tumors was dramatically reduced in antibiotics-treated or GF mice[5]. In antibiotics-treated mice, platinum adducts to tumor-cell DNA were formed at a level comparable to that observed in control mice, however, already at 48 hour post-treatment there was a significant decrease in DNA damage and cytotoxicity. Antibiotics treatment of mice largely suppressed all the gene expression modification induced in the tumor by oxaliplatin. In antibiotics-treated mice tumor-infiltrating myeloid cells failed to produce reactive oxygen species (ROS) via the NADPH oxidase NOX2. ROS are needed for the oxaliplatin antitumor effect. Thus, the microbiota affects oxaliplatin early tumor genotoxicity by systemically priming tumor-associated myeloid cells for ROS production.

The effects of microbiota deprivation in the response to CpG-ODN and platinum compounds were evident at very early times following treatment, suggesting that tumor-associated myeloid cells were primed for responsiveness to therapy by the preexisting microbiota composition. However, chemotherapy and radiation therapy in addition to their antitumor effect also induce damage of the intestinal mucosa affecting mucosal permeability and inducing dysbiosis and bacterial transmucosal translocation. Adoptive

transfer of tumor-specific cytotoxic $CD8^+$ T cells is an efficient therapy for cancer both in mice and in the clinic[12]. For best survival of the T cells and effectiveness of the transfer, lympho- and myelo-ablation are necessary[13]. Both in human patients and in mice, total body irradiation (TBI) improves therapy efficacy by increasing DC activation and homeostatic cytokine levels[13]. Due to the mucosal damage effect of TBI, commensal gut bacteria were found to infiltrate the mesenteric lymph nodes of irradiated animals and elevated endotoxin levels were observed in their sera[13]. The ability of TBI to improve tumor regression induced by adoptive T-cell transfer was reduced in animals treated with broad spectrum antibiotics, by neutralization of serum LPS using polymyxin B, or in mice genetically deficient in CD14 or TLR4 that are unable to respond to LPS. Administration of LPS or LPS-containing serum from irradiated animals to non-irradiated lymphopenic mice was able to enhance the number and function of transferred $CD8^+$ T cells, leading to long-term cure of mice with large transplanted tumors[13].

Cyclophosphamide (CTX) treatment of conventionally raised animals induces dysbiosis and mucositis. Due to the mucosal damage, $Gram^+$ bacteria translocate into the mesenteric draining lymph nodes, prime pathogenic effector Th17 cells and memory Th1 cells, all of which were not observed in microbiota-depleted mice[3]. Thus, the activation of antigen-presenting cells and the subsequent induction of antitumor immune responses by chemotherapy-induced immunogenic cell death not only depend on the release of endogenous mediators of inflammation as previously shown[14], but also on the priming and/or activating effects mediated by commensal bacteria and/or by their products.

In the past few years there has been very promising progress in the therapy of melanoma, kidney and lung cancers in terms of boosting the patient's immune response against the tumor using immune checkpoint inhibitors such as antibodies blocking the CTLA4 or PD-1 checkpoint receptors[15]. The role of the commensal microbiota in modulating the response to cancer immunotherapy, immunogenic chemotherapy and adoptive T-cell transfer suggest the possibility that the microbiota may also modulate the clinical effectiveness of this new class of anti-cancer drugs.

In conclusion, there is a considerable body of evidence, both in humans and in experimental animals, that the commensal microbiota – bacteria, fungi, and viruses – exerts important effects on carcinogenesis, tumor progression, and the response to therapy. The effect of the microbiota on cancer can be local, situated at the level of the organism barriers in which cancer originates, or can be systemic, through the physiological communication of the organism and the microbiota through intact membrane or following alteration of barrier permeability in pathology. While many mechanisms of the local effects have been characterized in recent years, our understanding of the systemic effects is currently much more rudimental. A detailed understanding of these mechanisms both in experimental

animals and in humans will teach us how to target them therapeutically and could bring much progress in cancer prevention and treatment.

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Specialty and Present Interest:
Microbiome, Inflammation and Cancer Therapies

COMBINATION IMMUNOTHERAPY BASED ON THE CANCER CELL'S CHARACTERISTICS AND PATIENTS' IMMUNE STATUS

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Pretreatment immune-status in tumor microenvironments varies among cancer patients. High infiltrations of $CD8^+$ T-cells, $CD3^+$ T-cells, $FOXP3^+$ T-cells and B-cells in cancer tissues of stage I-II colon cancer patients were found to be significantly correlated with favorable prognosis after surgery. $FOXP3^+$ T-cells in colon cancers may contain helper T-cells which may be partly activated by intestinal bacteria. High $FOXP3^+$ T-cells are generally correlated with poor prognosis in other types of cancers (e.g. lung cancer, oral squamous cancer, and cervical cancer which we have evaluated). Pretreatment immune-status in blood also varies among patients. We measured more than 50 cytokines and chemokines in blood of patients with various cancers by using multiplex analysis. High pretreatment levels of some cytokines were found to correlate with poor prognosis after various cancer immunotherapies including peptide vaccines and antigen pulsed dendritic cell vaccine. In the peptide vaccine protocol, delayed clinical effect (delayed separation of Kaplan-Meier curve) was not observed in the patients with high blood cytokine levels. Multivariate analyses showed that some cytokines were significant independent factors along with induction of tumor antigen specific T-cells and IgG Abs for post-immunotherapy prognosis. Therefore, the evaluation of pretreatment immune-status in tumor and blood may be useful for cancer therapies to predict prognosis and treatment response, thereby selecting appropriate patients and applying personalized therapy.

The immune-status may be defined by balance between positive and negative pathways in the anti-tumor T-cell responses which are regulated by cancer cell characteristics (e.g. immunogenic mutations, oncogene activation), patients' immune-reactivity (e.g. HLA type, polymorphism of immune-regulating molecules) and environmental factors (e.g. smoking,

diet, intestinal microbiota, previous history of infection). The positive pathway, induction of tumor antigen specific T-cells, may be regulated by quantity and quality of immunogenic mutated antigens derived from DNA mutations as well as immunogenic shared antigens. We found that tumor infiltrating T-cells (TIL) frequently recognize mutated unique peptide antigens derived from passenger mutations in melanoma[1,2]. We also found that immune responses to frameshift mutations occurs in patients with microsatellite instability positive (MSI^+) colon cancers having abundant DNA mutations due to dysfunction of DNA mismatch repair enzymes, and also high $CD8^+$ T-cell infiltrations in tumors[3]. In cervical cancer, TIL frequently recognize HPV oncoviral proteins, E6 and E7. Enhancement of the anti-tumor T-cell induction may be achieved by release of immunogenic antigens by induction of immunogenic cancer cell death (e.g. chemotherapy, molecular targeted therapy, radiation), stimulation of DC with strong adjuvants (e.g. TLR3, STING stimulators), and immune-checkpoint blockade (e.g. anti-CTLA-4 Ab, PD-1/PD-L1 blocking Ab)

In the negative pathway, suppression of anti-tumor immune responses, constitutive activation of various signaling pathways caused by genetic alternations such as activated oncogenes in cancer cells triggers multiple immunosuppressive cascades via production of immunosuppressive cytokines such as TGF-β, IL10, IL6 and VEGF, and via generation of immunosuppressive cells such as Tregs, DCregs and MDSCs. In human melanoma, BRAF/MAPK, STAT3, or Wnt/β-catenin is constitutively activated in some patients, and their activation generates immunosuppressive condition such as impaired function of DC and T-cells partly due to production of immunosuppressive cytokines and chemokines such as IL6, IL10, VEGF and CCL2[4,5]. These cytokines generate various immunosuppressive cells including MDSCs, tDCs, and Tregs through activation of STAT3 in the immune cells[6]. Increase of TGF-β in tumor microenvironments decreases accumulation of anti-tumor T-cells in tumors through impairment of DCs, increase of MDSCs and Tregs in tumors and sentinel lymph nodes[7]. TGF-β induced epithelial to mesenchymal transition (EMT) of melanoma may result in enhanced metastasis through snail induced production of immunosuppressive cytokines and chemokines such as TSP-1, TGF-β and CCL2 along with the increased cancer cell motility[8]. In heal and neck squamous cancers, expression of CCL22 and CCR4 in cancer cells not only enhances their lymph node metastasis, but also induce M2-like macrophages producing CCL22 which further recruit $CCR4^+$ Tregs in tumor and sentinel lymph nodes[9]. In human ovarian cancers, NF-κB is constitutively activated and generates immunosuppressive microenvironments such as impaired DC function and accumulation of immunosuppressive MDSCs partly due to high production of IL6, and IL8[10]. Constitutive activation of NF-κB in cancer cells may also inhibit IFN-α production by pDC via expression of ILT7 ligand[11], which may result in

decrease of anti-tumor T-cell induction.

Administration of various inhibitors against these oncogene products such as MAPK, β-catenin and NF-κB are useful for reversal of the immunosuppressive conditions in tumor microenvironments through reversal of impaired DC function and inhibition of immunosuppressive MDSCs and Tregs by acting on both cancer cells and immune cells in mouse models implanted with human cancer cells[4,5,10]. In syngeneic murine tumor models, either NK-κB dependent IL6 producing tumor or non-IL6 producing tumor, administration of NF-κB inhibitors enhanced induction of anti-tumor T-cells accompanied by restoration of T-cell stimulatory activity of DCs. However, administration of inhibitor alone was not sufficient to inhibit tumor growth because of local negative feedback (adaptive resistance) by PD-1/PD-L1 interaction in tumor microenvironments. Combined administration of anti-PD-L1 Ab with the NF-κB inhibitors resulted in synergistic inhibition of tumor growth. Therefore, we are attempting to develop combination immunotherapy using appropriate signal inhibitors, immune checkpoint blockers, and immunostimulating adjuvants. Some of the combinations have demonstrated superior anti-tumor activities. Personalized combination immunotherapy based on the immune-status of patients is a promising strategy[12].

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Specialty and Present Interest:
Tumor Immunology, Tumor Microenvironment, Cancer Immunotherapy

THE IMMUNE CONTEXTURE OF HUMAN CANCERS: SHAPING OF CLINICALLY EFFICIENT LANDSCAPES

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Tumors grow in a complex tissue where vascular and lymphatic vessels, inflammatory and immune cells, present at different levels, influence cancer spreading and patient's clinical outcome. The density, location and functional orientation of tumor-infiltrating lymphocytes from the immune contexture which composition is strongly correlated with patient's survival[1)]. Thus, in the vast majority of cancers, a high infiltration of Th1/cytotoxic T cells is associated with good disease free and/or overall survival[1)], both in primary and metastatic sites, as exemplified in colorectal cancer[2,3)]. There are however, exceptions to this rule as in Hodgkin lymphoma[4)] and renal cell cancer[5)] where a strong infiltration of $CD8^+$ T cells is associated with a poor prognosis. Moreover, increasing evidence support that the potential beneficial effect of Th1/cytotoxic T cells may be hampered by other components of the tumor microenvironment, such as regulatory T cells, or myeloïd derived suppressor cells as well as by tumor cell expression and production of immunossuppressive molecules (reviewed in 6).

The requirements for shaping a clinically efficient immune landscape were analyzed. We found that the site of education of adaptive immune cells was essential. Thus, in tumors with high density of Tertiary Lymphoïd Structures (TLS), $CD8^+$ T cells and B cells were recruted, activated and educated without passing through the tumoral immunosuppressive milieu and become effector and antibody producing cells, with a positive impact on patient's survival[7,8)]. $CD8^+$ T cells, educated outside TLS were associated with a bad prognosis. It was particularly exemplified in Non Small Cell Lung Cancer (NSCLC) and Colorectal cancer (CRC). In NSCLC, high density of $CD8^+$ T cells correlate with longer disease free survival (DFS) and overall survival (OS) both in early[10)] and

advanced stage (up to stage III)[7] tumors. Mature Dendritic Cells (DC), expressing the DC-Lamp marker, were almost exclusively found in TLS[10,11]. The combined densities of $CD8^+$ T cells and DC-$Lamp^+$ cells identified cohorts with the highest (Lo/lo) and the lowest (Hi/Hi) risk of relapse[7,10] suggesting that the enumeration of TLS-associated mature DC improves the prognostic impact of $CD8^+$ T lymphocytes. Indeed, the analysis of the cohort of patients with high densities of intratumoral $CD8^+$ T cells, stratified according to the density of DC-$Lamp^+$ cells identified a cohort of patients with high $CD8^+$ T cells but low numbers of DC-$Lamp^+$ cells and few TLS, with short DFS and OS[7]. These data suggest that when $CD8^+$ T cells are not likely educated in TLS, they may not be active in controlling tumor spread[10]. In CRC, in which the paradigm of the pronostic impact of the infiltration by $CD8^+$ memory T cells and a Th1 orientation has been established[1,12,13] leading to the definition of the Immunoscore[14,15], tumors in which the gene encoding CXCL13, a chemokine involved in the formation of tumor-associated TLS[11], is deleted identifies a group of patients with poor prognosis[16].

In Renal Cell Cancer (RCC), as in Lymphomas[16,17] a high density of $CD8^+$ T cells correlates with shorter DFS and OS in primary[5] and metastatic[3] patients. We revisited this surprising findings by analyzing cohorts of primary and metastatic clear cell RCC (ccRCC) and not only confirmed it on larger cohorts of primary ccRCC, but also identified a group of tumors where there were high densities of DC $Lamp^+$ cells, lacking characterisitics of mature DC and few TLS. In these tumors, $CD8^+$ T cells are associated with poor prognosis[18]. Since the respective positive and negative prognostic impacts of $CD8^+$ T cells in CRC and RCC are found in metastatic locations in the lung[3,18,19], it suggests that the tumor cells, rather than the organ, shape the immune contexture.

If the tumor cells shape their immune microenvironment molecular subgroups of tumors from the same origin should identify different immune infiltrates. We established immune metagenes, based on the selective expression of genes in purified immunological populations, and applied them to larger cohorts of ccRCC and CRC[6]. A cohort of primary tumors of metastatic ccRCC has been classified according to tumor-associated molecular typing[20]. Four groups were identified, group 4 being the one with the worst prognosis in terms of survival[21]. Group 4 appeared to be the one with the highest expression of immune, inflammatory angiogenic and myeloïd cell attracting genes and where there was a high density of $CD8^{++}$ and $PD1^+$ lymphocytes as well as $PD\text{-}L1^+$ tumors[20]. In CRC, several molecular subgroups have been defined[21-28]. The application of the immune metagenes to molecular subgroups identified a cohort with microsatellite instability and the best prognosis. Another subgroup had a strong Th1/$CD8^+$ signature together with angiogenic and myeloid signatures and was associated with a poorer prognosis (E. Becht et al., in preparation). The analysis of large collections of colorectal and renal cell cancers, classified

according to tumor cell specific signatures, revealed an association of tumoral subtypes with immune signatures correlating with good or bad prognosis. The analysis of representative tumor cell lines revealed that the malignant cells express and produce the molecules found in subtypes of patient's tumors that modulate the immune contexture. The identification of these immunomodulatory tumoral subtypes is essential to guide the exploding field of immunomodulatory therapies.

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Specialty and Present Interest:
Tumor Immunology, Colorectal Cancer, Renal Cell Cancer, Tumor Microenvironment

MEDIATORS OF TUMOR INDUCED IMMUNE SUPPRESSION IN MALIGNANT MELANOMA

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Tumour-induced immune dysfunction is a serious challenge to immunotherapy for cancer. Immune suppressive mechanisms do not only affect the tumor microenvironment, but also result in decreased systemic immunity. An intact adaptive and innate cellular immunity are keys to the success of immunotherapy of cancer, why methods to restore immunity in patients with cancer are attractive to combine with immunotherapy. This is particularly important in adoptive T cell therapy with Tumor Infiltrating Lymphocytes (TIL), to avoid that *ex-vivo* expanded TIL cells are inactivated when re-injected into an immune suppressive environment. At Cancercenter Karolinska we treat patients with advanced malignant melanoma in the closed clinical trial MAT01[1)] and the ongoing trial MAT02 (ClinicalTrials.gov: NCT01946373) with a combination of TIL cells and autologous Dendritic Cells (DCs) derived from the same tumor as the TIL cells were isolated from. Methods are being developed to prolong the life span of injected TIL cells and to make these resistant to the immune suppressive mechanisms in the patient. We have focused on methods to counteract the central role that myelomonocytic cells and their products have in immune suppression. It is well established that tumor-associated macrophages, eosinophils, neutrophils and myeloid-derived suppressor cells (MDSC's) are of major importance in tumor induced immune suppression. In particular MDSC's secrete a broad repertoire of inflammatory mediators providing them with powerful tools to inhibit tumor reactive T cells and natural killer cells. These immune suppressive mediators include free oxygen radicals including reactive oxygen species (ROS) and nitric oxide (NO), arginase, indoleamine 2,3-dioxygenase, prostaglandins, the pro-inflammatory heterodimer S100A8/9 and cytokines, such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and

transforming growth factor-beta (TGF-β) which all have proven potent in suppressing anti-tumour cellular immunity (for review see 2). Our studies have focused on the monocytic MDSCs ($CD14^+HLA\text{-}DR^{lo/-}$) in peripheral blood of patients with advanced malignant melanoma[3-6]. The characteristics and suppressive properties of these mo MDSC's are summarized in table 1. These mo MDSC suppress T cell and NK functions *ex vivo* and correlate with lower numbers of antigen-specific T cells, but the exact mechanisms underlying this effect is unclear. We have recently analyzed *in vitro* the effects of tumor-derived factors in inducing phenotypic and functional alternations on healthy human monocytes or $CD34^+$ hematopoietic stem cells (HSCs) (3, 4 and unpublished data). This was done by co-culturing monocytes or $CD34^+$ hematopoietic stem cells (HSCs) with early-passage tumor cells isolated from metastatic lymph-nodes or addition of tumor-conditioned medium. Our results demonstrated that tumor-derived factors promoted monocytes to acquire an MDSC-like phenotype and suppressive functions of T cells and NK cells where the COX-2/PGE2 pathway played a dominant role. Mouse models are being explored to test the *in vivo* relevance of targeting MDSC and factors responsible for their expansion and effector functions. This was exemplified by a murine model, where 4T1 mammary carcinoma tumor cells were disabled from cyclooxygenase-2 (COX-2) production and subsequently their *in vivo* NK cytotoxicity was evaluated by live imaging[4].

MDSCs and regulatory T cells (T reg) constitute an important target for cancer immune therapy, and new methods to deplete cancer patients from these inhibitory cells will lead to better clinical responses. We have recently studied the effect that treatment with the check-point inhibitor ipilimumab, which targets the CTLA-4 molecule, has on MDSC and T reg cells in patients with advanced malignant melanoma[7]. Our results, as summarized in table 2, showed that T reg's are depleted in ipilimumab treated patient. As a new finding we observed an almost total decrease in the frequency of granulocytic MDSCs, while monocytic MDSC were not affected by this treatment. In ongoing experiments we are now analysing the mechanisms behind this drastic effect of CTLA-4 blockade on granulocytic MDSCs, and also ask to what extent analysis of MDSCs and their products (iNOS,

Table 1 Characteristics of monocytic MDSCs as studied at Cancercenter Karolinska (From references 3-6 and unpublished results)

- **CD14+HLA-DR- MDSC (moMDSC)** are increased in melanoma patients and strongly suppressive of T cell proliferation and IFNγ production
- Increase in active disease and is associated with disease course.
- Several mechanisms (**oxidative stress, Arginase 1, PGE2 , TGFβ)** contribute to suppression.
- PGE2 production and STAT-3 signaling are involved in the T cell suppression mediated by $CD14^+$ MDSC from advance stage melanoma patient
- Early-passage melanoma cells can induce MDSC-like cells with potent ability to suppress autologous T cells through COX-2/PGE2 production
- moMDSC are also suppressive for NK cells

Arginase) can be used in clinical practise to predict responsiveness to checkpoint inhibitors.

Reactive oxygen species (ROS) is another product of MDSCs which severely can impair cancer immune therapy. Oxidative stress produced by MDSCs and other cells in the tumor stroma can severely impair the function of both T cells and NK cells, leading to decrease in their signal transducing capacity and at high concentrations to apoptosis[8, 9]. In ongoing experiments we are "arming" T cells by gene transfer of the anti-oxidant enzymes catalase, which catalyzes H2O2 to be enzymatically reduced to water and oxygen, thereby protecting the T cells from functional defects or apoptosis induced by ROS. We have shown that T cells transduced with bicistronic construct expressing both catalase and a chimeric antigen receptors (CAR) are able to maintain functional anti-tumor activities even when confronted with high concentrations of H_2O_2 as well as H_2O_2 producing cells, granulocytes and MDSC (Ligtenberg et al, submitted for publication). Also, the protection offered to the CAR-CAT bicistronic construct transduced T cells was found to be extended to bystander cells, allowing for NK cells to functionally eliminate their K562 model cells under H_2O_2 induced oxidative stress.

Table 2 Effect of ipilimumab treatment on Treg and MDSC in patients with advanced malignant melanoma (From reference 7 and unpublished results)

- An initial increase in Treg´s followed by a significant decrease at week 9 before the second dose.
- Granulocytic MDSCs decrease strongly already after one dose at week 3.
- No change in the frequency of monocytic MDSCs.
- CD14 positive iNOS expressing and CD14 negative Arginase expressing myeloid cells are reduced as a result of treatment.

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THERAPEUTIC STRATEGIES BASED ON OVERCOMING IMMUNE RESISTANCE MECHANISMS WITHIN THE TUMOR MICROENVIRONMENT

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1. Characterization of the T cell-inflamed and non-T cell-inflamed tumor microenvironment.

Based on the lack of correlation between the induction of anti-tumor T cell responses as measured in the blood and clinical responses in melanoma, we began over 10 years ago to probe the tumor microenvironment for predictive biomarkers for clinical response. Two major categories of melanoma metastases have been observed based on gene expression profiling and confirmatory assays. One subgroup of patients has an inflamed phenotype that includes expression of chemokines, T cell markers, and other immunoregulatory factors including a type I IFN signature. In contrast, the other major subset lacks this phenotype and appears to display immune "exclusion"[1-3]. The T cell-inflamed tumor microenvironment subset shows the highest expression of negative regulatory factors, including PD-L1, IDO, and FoxP3$^+$ Tregs[4], and evidence for T cell-intrinsic anergy has also emerged aided by a recently defined functional role of EGR2[5,6]. In addition, the mechanism of induction of these inhibitory mechanisms has been elucidated—PD-L1 and IDO are induced by IFN-γ, and Tregs are largely recruited by the chemokine CCL22, both being produced by activated CD8$^+$ effector T cells[7]. Among the T cells within the tumor site are CD8$^+$ T cells specific for defined tumor antigens[8-10]. Moreover, these T cells represent the starting point for tumor-infiltrating lymphocyte (TIL)-based adoptive therapy for melanoma[11]. Thus, the T cell-inflamed tumor microenvironment appears to represent a situation of chronic T cell activation trapped in the negative feedback phase of an adaptive immune response. In contrast, the non-T cell-inflamed tumors appear to have minimal communication with the host immune response and demonstrate what appears to be immune exclusion.

2. Immunotherapeutic interventions targeting the T cell-inflamed tumor subset.

The optimal immunotherapeutic interventions may be distinct in the T cell-inflamed versus non-T cell-inflamed tumor phenotypes[12]. As a predictive biomarker, early data analyzing the clinical responders in melanoma to vaccines, high-dose IL-2, the anti-CTLA-4 mAb ipilimumab, and anti-PD-1 mAbs largely fall within this group[13-18]. These data argue that the tumor microenvironment must support chemokine-mediated T cell trafficking into the tumor microenvironment for current immunotherapies to succeed.

The presence of defined immune-inhibitory pathways in these inflamed tumors argues that blockade of these pathways might restore effective T cell function and lead to improved tumor control. Strategies to block PD-1/PD-L1 interactions, inhibit IDO function, deplete Tregs, and reverse T cell anergy have all shown success in preclinical models[12,19-21]. Anti-PD-1 and PD-L1 mAbs have shown remarkable clinical responses in metastatic cancer patients, and one of these (pembrolizumab) was FDA approved in September, 2014 for melanoma[22,23]. Early clinical biomarker data have suggested again that majority of responding patients have a baseline T cell infiltrate, supporting our working model[18]. The presence of multiple inhibitory mechanisms in the same tumor microenvironment argues that combination therapies may be advantageous. Preclinical data have indicated synergy between anti-CTLA-4 +/- anti-PD-L1 +/- IDO inhibition, among others[21]. The mechanism of synergy is striking, as it correlates with a marked improvement of IL-2 production and proliferation of tumor-infiltrating $CD8^+$ T cells[21]. Clinical translation of these combination immunotherapies is promising and ongoing, with response rates of approximately 50% in early studies of anti-CTLA-4 mAb combined with anti-PD-1 or with an IDO inhibitor[24,25]. Taken together, these observations have generated tremendous enthusiasm that manipulation of immune regulatory pathways may ultimately translate into clinical activity in the majority of patients having the T cell-inflamed tumor microenvironment phenotype.

3. Understanding of innate immune sensing that gives rise to the T cell-inflamed phenotype—importance of the STING pathway.

We believe that a next major hurdle in the cancer immunotherapy field is to understand why a major subset of patient tumors excludes the host immune response and fails to generate the T cell-inflamed signature. One approach has been to work towards identification of the innate immune sensing pathways that mediated spontaneous T cell priming against sterile tumors. Based on the presence of a type I IFN signature in the inflamed tumors, we utilized mouse models to determine whether type I IFN signaling was required upstream from endogenous T cell priming against tumor antigen. Indeed this was the case, and the effect was mediated though activity on the $CD8\alpha^+$ subset of dendritic cells

(DCs) that carry out antigen cross-presentation[26-28]. Moving upstream to identify the requisite pathways for induction of IFN-β production by DCs, we uncovered a critical role for the STING pathway[29]. STING-deficient mice failed to control immunogenic tumors, were defective in spontaneous T cell priming, and showed absent IFN-β production by tumor-infiltrating DCs. The STING pathway is involved with cytosolic DNA sensing, and indeed we identified tumor-derived DNA within the cytosol of DCs *in vivo*. A major implication of these observations is that the non-T cell inflamed tumors likely have failed to engage the host innate immune pathways, in particular STING. A corollary is that deliberate activation of the STING pathway may be an attractive novel therapeutic consideration.

4. Identifying molecular correlates involved in T cell exclusion.

An additional approach for determining mechanisms of immune evasion in the non-T cell-inflamed tumors has been to analyze molecular features of patients correlating with the presence or absence of this phenotype, combined with mouse models for mechanistic studies. Three hypothetical sources of inter-patient heterogeneity are being evaluated: somatic differences at the level of the tumor cells, germline polymorphisms in immune regulatory genes, and environmental differences in the composition of the intestinal microbiome. Preliminary data are supporting functional relevance of all three of these variables. Within the tumor cells, we have identified the first oncogene pathway which

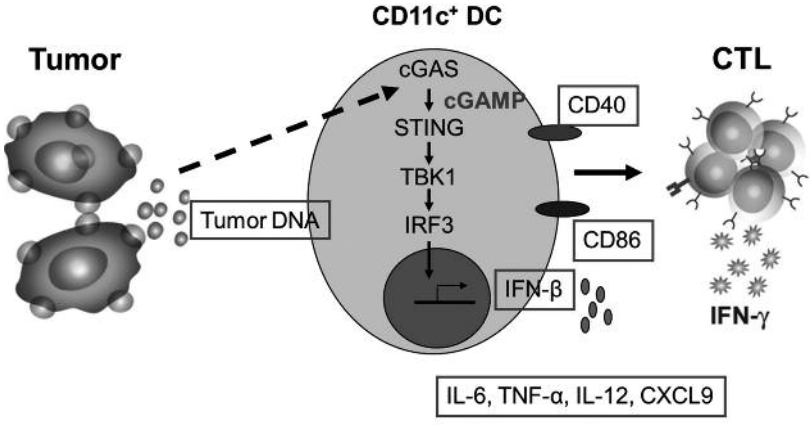

Figure 1 Identification of the STING pathway as a major mechanism of innate immune sensing in cancer.
Current evidence indicates that tumor-derived DNA can become taken up and localized to the cyctosol of tumor-infiltrating DCs, where cGAS becomes activated. This generates intracellular cGAMP, which promotes STING aggregation and downstream phosphorylation of TBK1 and IRF3. This leads to transcription of the IFN-β gene and also results in a broad DC activation profile. These activated DCs then are capable of cross-priming $CD8^+$ T cells which ultimately home back to the tumor and support tumor growth control.

appears to mediate exclusion of the host immune response, which is tumor-intrinsic activation of the β-catenin pathway. Germline polymorphisms of high statistical significance have been identified that correlate with high presence of T cell infiltration. Finally, mouse preclinical experiments have indicated that altering only the intestinal microbiota can profoundly influence immune-mediated tumor control *in vivo*. A thorough analysis of these factors in patients with a range of advanced cancers should be performed to uncover similar mechanisms beyond melanoma. Each of these mechanisms can be envisioned to give rise to new therapeutic interventions.

5. Future perspectives: potential strategies to promote immunity in non-T cell-inflamed tumors.

In contrast to the T cell-inflamed melanomas, new paradigms may be needed to promote de *novo* inflammation in cases of the non-T cell-infiltrated tumor microenvironment. Based on recent data implicating the STING pathway and endogenous type I IFN production in spontaneous immune priming, STING agonists are being evaluated to promote activation of this pathway. Tremendous anti-tumor activity has been observed in preclinical experiments, supporting plans for clinical translation. Increasing IFN-β abundance in the tumor microenvironment also is an effective strategy. Coupling IFN-β to tumor-targeting mAbs has shown interesting activity in mouse models that works through an immunologic mechanism[30]. Direct administration of high doses of IFN-β within the tumor microenvironment also is therapeutic, although the majority of this activity is through an anti-angiogenic effect[31]. Recent work has suggested that targeted radiation to the tumor also promotes STING pathway activation and type I IFN production *in vivo*[32].

Additional therapeutic strategies can be envisioned based on data emerging from the genomic analysis of patient specimens. Our current data already have suggested that pharmacologic strategies to inhibit the β-catenin pathway may provide one means of reversing an immune-inhibitory oncogene pathway. It also might be feasible to pursue pharmacologic approaches to functionally mimic the effect of favorable germline polymorphisms. Finally, manipulation of the intestinal microbiome to facilitate anti-tumor immunity and potentiate the T cell-inflamed tumor microenvironment phenotype also is attractive to consider, for example through development of appropriate probiotics. Thus, as these molecular mechanisms continue to be unraveled, novel therapeutic strategies to support natural T cell responses against tumors can be envisioned. Combining such interventions with blockade of immune inhibitory pathways ultimately may be necessary for maximal therapeutic efficacy at the population level.

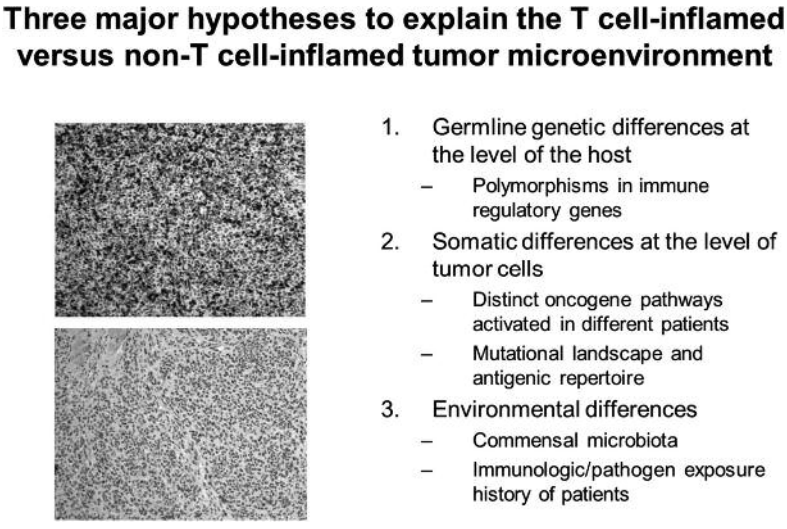

Figure 2 Hypothetical mechanisms of inter-patient heterogeneity that could contribute to the presence or absence of a spontaneously generated T cell-inflamed tumor microenvironment. Genomics approaches are being pursued to analyze germline polymorphisms, tumor somatic changes, and intestinal microbiome species that may associate with the presence or absence of a T cell-inflamed tumor microenvironment in individual patients. Integration of these genomics platforms should provide a compresensive assessment of molecular correlates to host anti-tumor immune responses, and also identifiy new therapeutic approaches for intervention. Inasmuch as the T cell-inflamed tumor microenvironment is associated with clinical response to immunotherapies, it is envisioned that a multivariate predictive biomarker algorithm will ultimately be created from these data.

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REPROGRAMMING THE TUMOR MICROENVIRONMENT FOR EFFICIENT TUMOR REJECTION

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To date, immunotherapy of cancer has met limited clinical success. Responsive cases, however, demonstrate that immunotherapy can be a powerful approach. They also show that for improvement of immunotherapy the mechanisms of tumor rejection need to be explored in more detail. Clinical and experimental studies have identified several mechanisms that prevent tumor rejection. Tolerance mechanisms including regulatory T cells (Tregs) play an important role as they result in impaired activation of tumor-specific T lymphocytes. Of crucial importance are also insufficient infiltration of T cells into tumors and an inhibitory tumor microenvironment.

Danger signals enhance T cell infiltration.

We have reported that the usually aberrant phenotype of the tumor vasculature significantly contributes to the lack of infiltration. An aberrant vasculature is also characteristic for Rip.Tag mice that develop autochthonous insulinomas in a multi-step process closely resembling the clinical situation of human pancreatic neuroendocrine tumors. Vaccination or transfer of tumor-specific T lymphocytes failed to eliminate the tumors, because the aberrant tumor endothelium formed a barrier against cell infiltration. We have observed that the endothelial barrier could be overcome by induction of an inflammatory microenvironment in the tumor by danger signals, e.g. by irradiation or immunostimulatory TLR ligands. Both of these auxiliary treatments resulted in normalization of the tumor vasculature, expression of adhesion molecules such as VCAM1 on vessels, efficient T cell infiltration, and tumor eradication[1,2)], see Figure 1.

These findings raised two major questions, namely 1. the mechanisms by which irradiation and TLR ligands exert their dramatic effects, and 2. whether or not vessel normalization is important for T-cell infiltration.

Vessel normalization.

In order to study a potential role of vessel normalization, we performed gene expression profiling and found regulator of G protein signalling 5 (RGS5) to be strongly expressed on the aberrant tumor vasculature, but largely absent on normal vessels. RGS5 k.o. mice were produced and crossed with RipTag5 mice. The RGS5-/- x RipTag mice developed insulinoma with about the same kinetics as RGS5-positive RipTag mice. Detailed analysis revealed that RGS5-negative tumors exhibited a normalized vasculature. In addition, tumor hypoxia was decreased. Importantly, immunization or adaptive T cell transfer resulted in T cell infiltration and survival of the animal[3]. These data are schematically presented in Figure 2.

These results demonstrate that normalization of tumor vessels is sufficient to permit T cell infiltration without the requirement for danger signals. Probably the decreased hypoxia is of importance as it is known to influence T cell functions.

Next, we investigated the mechanisms by which danger signals might control T cell infiltration and tumor rejection.

TLR ligands.

We have observed that labelled CpG binds to tumor associated macrophages[2]. In order to see if this binding and subsequent activation of the macrophages was important, we depleted macrophages with clodronate liposomes and observed that now tumor rejection was impeded. Mechanistically, polarization of immunosuppressive M2-like tumor macrophages towards immunostimulatory M1-like $iNOS^+$ macrophages was found to be required for tumor rejection.

Local low dose radiation.

With regard to radiation , local radiation with a low dose of 2 Gy combined with T cell transfer was sufficient to support vessel normalization, infiltration of T cells and tumor rejection. Interestingly, macrophages were again crucial for this process, in particular the iNOS positive M1 polarized macrophages, because blocking of iNOS *in vivo* with the specific inhibitor 1400w abrogated tumor rejection[4].

From Tregs to eosinophils.

In another set of studies we adressed the question as to how efficient Treg depletion would affect spontaneous tumor immunity. 90%depletion of Tregs (but not 70% depletion)

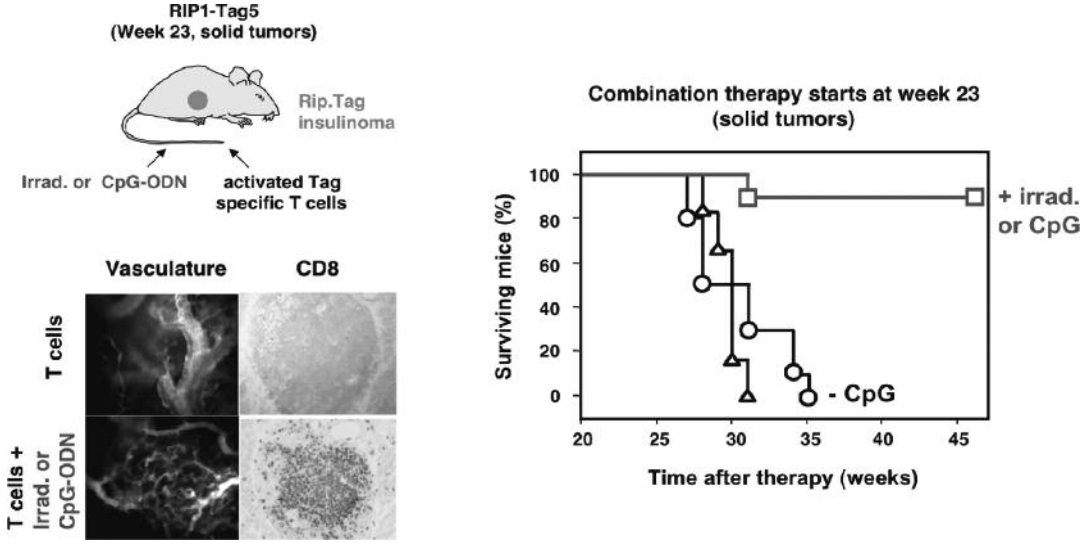

Figure 1 T cell infiltration and tumor rejection after induction of inflammation by irradiation or TLR stimulation.
RipTag mice carrying established tumors were irradiated or treated with CpG-ODN and then transferred with activated tumor-specific T cells. Irradiation or CpG-ODN treatment lead to vessel normalization and T cell infiltration (left part), resulting in survival of the animals (right hand part).

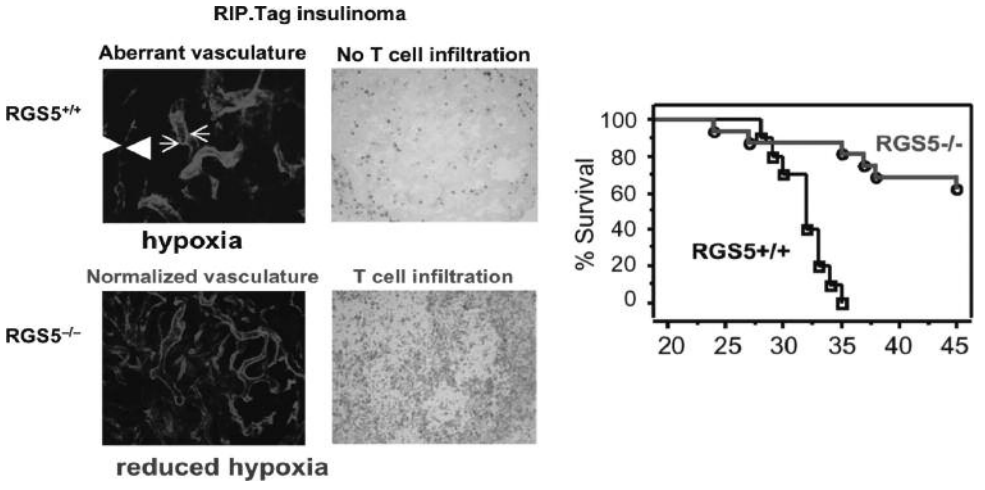

Figure 2 RGS5 (regulator of G protein signalling) knockout results in normalization of the tumor vasculature, T cell infiltration and increased survival.
The left hand part shows the typically aberrant vasculature of wild-type (RGS5+/+) RipTag insulinoma which prevents infiltration of tumor-specific T cells. In RGS5-/- mice the vasculature is normalized and permits strong T cell infiltration, thereby enhancing survival (right hand part).

in tumor bearing Foxp3.LuciDTR mice resulted in a cytokine storm and at the same time in activation of endogeneous tumor specific T cells, normalization of the tumor vasculature, CD8 T cell infiltration and tumor eradication[5]. Interestingly, not only T cells were found to infiltrate the tumor after Treg depletion but also eosinophils. Tumor eosinophilia is often observed in the clinic, but its function is a long standing question and so far only a matter of speculations[6,7]. Surprisingly, depletion of eosinophils by specific Siglec F antibody impaired tumor eradication. Cotransfer studies with activated eosinophils and T- cells revealed that eosinophils migrated into the tumor where they produced chemokines that attracted T cells into the tumor. Concommitantly the tumor macrophages were skewed towards M1 and the vasculature was normalized (unpublished data).

Conclusions.

The results presented hereindicate that different approaches of T cell mediated therapy of cancer function via the same mechanisms, namely reprogramming of the tumor microenvironment, with macrophage polarization towards M1 and vessel normalization, as depicted below in Figure 3. We suggest that for successful clinical cancer immunotherapy vaccination or adoptive T cell transfer should be combined with modulators of the tumor microenvironment, such as TLR ligands or local low dose tumor irradiation which

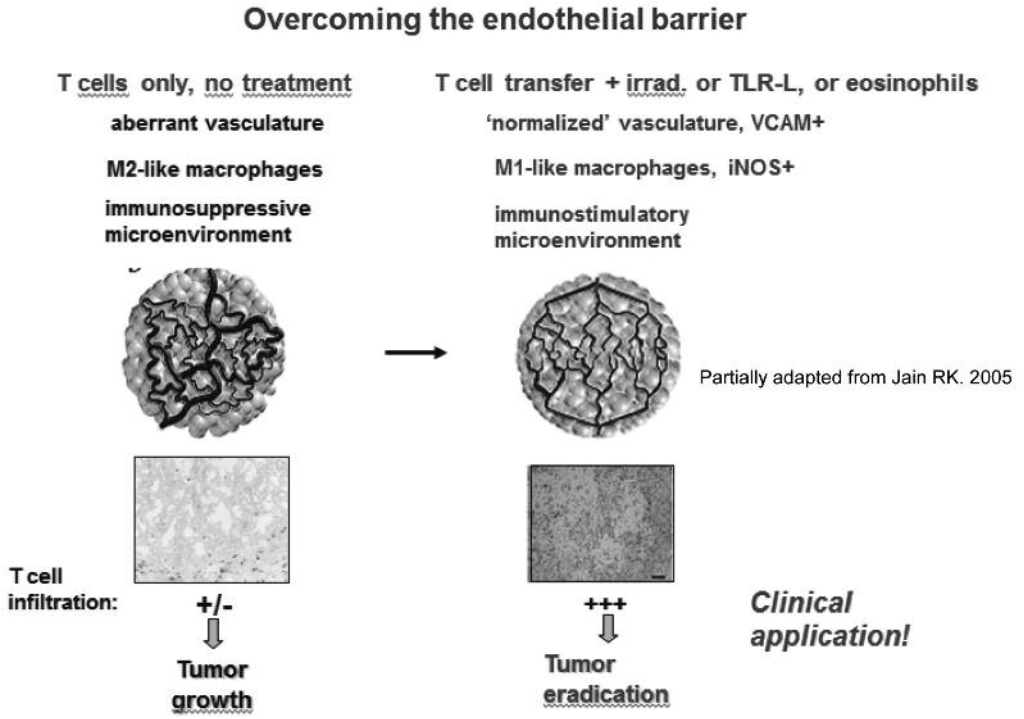

Figure 3

polarizes tumor macrophages, thereby enhancing T cell infiltration. Indeed, in preliminary patient studies, local irradiation of pancreatic tumors resulted in accumulation of iNOS$^+$ M1 macrophages and increased T cell infiltration[4]. Together, these observations open the way for novel therapeutic interventions.

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HARNESSING REGULATORY T CELLS IN CANCER IMMUNOTHERAPY

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Regulatory T cells (Tregs) hinder cancer immuno-surveillance and also prevent the induction of effective anti-tumor immunity. It is therefore becoming an important priority in the cancer immunotherapy field to find strategies for controlling Tregs, in addition to stimulating effector T cells by immunostimulatory signals including immune checkpoint blockade and antigen-specific cancer vaccines[1,2]. Indeed, depletion of Tregs enhances spontaneous and vaccine-induced anti-tumor immune responses, and the stimulation of Tregs by immunization with self-antigens induces enhanced chemically-induced primary tumor development and increased numbers of pulmonary metastasis following injection of transplantable tumor cells in animal models[3-7]. In humans, the presence of high numbers of Tregs or a low ratio of $CD8^+$ T cells to Tregs in tumor tissues is correlated with an unfavorable prognosis in various types of cancers[1,8,9]. Thus, an understanding of the detailed mechanisms of Treg suppression against tumor-antigen-specific T cells is crucial. Yet, how Tregs stably suppress self-(tumor)-antigen-specific $CD8^+$ T cells remains obscure.

We investigated proliferation, cytokine production, and the cell fate of self-(tumor)-antigen-specific $CD8^+$ T cells in peripheral blood mononuclear cells (PBMCs) when stimulated *in vitro* with self-antigen Melan-A/MART-1 (Melan-A) peptide in the presence or absence of natural $FoxP3^+CD25^+CD4^+$ Tregs. Melan-A-specific $CD8^+$ T cells activated with physiologically-stimulated Tregs underwent one cell division and stopped further proliferation. These Melan-A-specific $CD8^+$ T cells had an anergic phenotype (hypo-proliferative and cytokine hypo-producing upon TCR re-stimulation). Such anergic T cells possessed lower affinity T-cell receptors (TCRs) for the stimulating self-antigen than antigen-activated proliferating T cells. Importantly, Melan-A-specific $CD8^+$ T cells obtained

from Treg-absent or -present cell cultures had substantial differences in gene expression profiles. Particularly, Melan-A-specific $CD8^+$ T cells suppressed by Tregs were distinct from other T cells in being phenotypically naïve, e.g., CC chemokine receptor 7 (CCR7) positive, but expressing high levels of several co-inhibitory molecules, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). This unique phenotype enabled *in vivo* detection of hitherto elusive anergic T cells as a distinct T-cell subpopulation in healthy individuals, and revealed concurrent generation of anergic T cells and activated T cells in an immune response. Mechanistically, in accordance with the ability of Treg cells to down-modulate the expression of the co-stimulatory molecules CD80 and CD86 on antigen-presenting cells (APCs)[10,11], blockade of these molecules on APCs was able to drive low-affinity self-reactive T cells into anergy with similar phenotype and function to the anergic T cells observed following Treg suppression. Melan-A-specific $CD8^+$ T cells with this anergic phenotype were detected *ex vivo* in PBMCs from healthy individuals in contrast with the effector or memory phenotype of the majority of Melan-A-specific $CD8^+$ T cells in vitiligo, and therefore contributed to the tolerance of self-(tumor)-antigens[12]. This Treg-dependent induction of anergy is likely operating in tumor immunity, indicating the importance of controlling Tregs to allow the efficient activation of self-(tumor)-antigen-specific $CD8^+$ T cells.

To control Tregs, we attempted to find specific markers of tumor (malignant melanoma)-infiltrating Tregs. $FoxP3^+CD4^+$ T cells can be dissected into three subpopulations by the expression levels of FoxP3 and the cell surface molecules CD45RA and CD25: $FoxP3^{lo}CD45RA^+CD25^{lo}$ cells, designated naive or resting Tregs, $FoxP3^{hi}CD45RA^-CD25^{hi}$ cells, designated effector Tregs, which are terminally differentiated and highly suppressive; and $FoxP3^{lo}CD45RA^-CD25^{lo}$ non-Tregs, which do not possess suppressive activity but can secrete pro-inflammatory cytokines[2,10]. In melanoma tissues, Effector Tregs were predominant among tumor-infiltrating $FoxP3^+$ T cells and much higher in frequency compared with those in peripheral blood. These melanoma infiltrating effector Tregs specifically expressed CC chemokine receptor 4 (CCR4). With peripheral blood lymphocytes from melanoma patients, *ex vivo* depletion of $CCR4^+$ T cells (effector Tregs) and subsequent *in vitro* stimulation of the depleted cell population with the cancer/testis antigen NY-ESO-1 efficiently induced NY-ESO-1-specific $CD4^+$ T cells. Non-CCR4-depleted cells failed to induce these cells. The magnitude of the responses was comparable to those seen following total removal of $FoxP3^+$ Tregs by $CD25^+$ T-cell depletion. $CCR4^+$ T-cell depletion also augmented *in vitro* generation of NY-ESO-1-specific $CD8^+$ T cells in melanoma patients. Furthermore, *in vivo* administration of anti-CCR4 mAb markedly reduced the effector Treg fraction and augmented NY-ESO-1-specific $CD8^+$ T cell responses in an adult T-cell leukemia-lymphoma patient whose leukemic cells expressed NY-ESO-1.

Collectively, these findings indicate that anti-CCR4 mAb treatment is instrumental for evoking and enhancing anti-tumor immunity in cancer patients by selectively depleting effector Tregs[13]. We propose that selective reduction of effector Tregs, rather than whole Treg population can augment anti-tumor immune responses without significant autoimmunity, and CCR4 can be a possible target for selective Treg control.

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IMMUNOGENIC CELL DEATH IN CANCER THERAPY

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The supreme goal of anticancer therapy is the physical elimination of tumor cell by causing their death. In physiological settings, cell death occurs as a continuous byproduct of cellular turnover. This type of cell death is non-immunogenic or even tolerogenic, thus avoiding the induction of autoimmune disease. However, the iatrogenic cancer cell death elicited by radiotherapy and a specific class of chemotherapeutic agents (such as anthracyclines and oxaliplatin) can be immunogenic. Immunogenic cell death (ICD) involves subtle changes in the external surface of the plasma membrane, as well as the release of soluble immunogenic signals. These changes in the cell surface and the pericellular microenvironment must occur in a defined temporal sequence that constitutes a 'code' or 'key'. This 'code' is decoded by a series of receptors expressed by cells from the innate immune system, in particular dendritic cells (DC, the 'lock'). If all the changes accompanying immunogenic cell death are present and – presumably – occur in the right order, as well as at the correct intensity, they allow for the DC-mediated cross-presentation of tumor-specific antigens to T lymphocytes and for triggering of an efficient anticancer immune response[1-6]. Thus far, we have been able to identify four hallmarks of ICD.

Calreticulin exposure.

Immunogenic cell death is characterized by the pre-apoptotic cell surface exposure of calreticulin (CRT), which determines the uptake of tumor-associated antigens by DC. The precocious release of CRT is the result of an endoplasmic reticulum stress response (also called unfolded stress response) causing the translocation of CRT from the endoplasmic reticulum lumen to the cell surface. Tumors that lack CRT expression or that lack elements

of the CRT exposure pathway are relatively resistant to anticancer chemotherapy (as compared to normal control tumors) unless recombinant CRT protein is injected into the tumor[7-9].

ATP release.

During the apoptotic blebbing phase, dying cells can release adenosine triphosphate (ATP) through a pathway that is particularly efficient if the cells have undergone premortem induction of autophagy, likely due to a the autophagy-mediated increase of intralysosomal ATP stores. The release of ATP from apoptotic cells causes the P2Y2 purinergic receptor-mediated attraction of DC precursors into the tumor bed. ATP released from dying cancer cells also acts on another type or purinergic receptors, namely ionotropic P2RX7 receptors. P2RX7 receptor-dependent activation of the NLRP3 inflammasome in DC allows these cells to release interleukin-1β and hence to polarize tumor antigen-specific CD8 T lymphocytes towards a Tc1 cytokine pattern. Tumors that are autophagy deficient fail to elicit an anticancer immune response following treatment with anthracyclines or oxaliplatin and hence are resistant to anticancer chemotherapy with these agents. Moreover, mice that lack P2RX7 expression or mice in which P2Y2 has been blocked pharmaceutically fail to mount anticancer immune responses post-chemotherapy[10-17].

HMGB1 exodus.

At a post-apoptotic stage, as the plasma membrane undergoes permeabilization during secondary necrosis, the protein high mobility group box 1 (HMGB1) is released. Extracellular HMGB1 acts on toll-like receptor 4 (TLR4), the activation of which is required for the optimal presentation of antigens from dying tumor cells. This effect is likely obtained by virtue of the capacity of TLR4 to inhibit the lysosomal degradation of ingested tumor antigens. TLR4 ligation also provides a signal for the transcriptional upregulation of the gene coding for interleukin-β. Tumor cells that lack HMGB1 expression fail to elicit an anticancer immune response, and HMGB1-negative tumors become refractory to chemotherapy with anthracyclines or oxaliplatin. Moreover, HMGB1-expressing tumors transplanted to mice lacking TLR4 or its adaptor MYD88 fail to respond to chemotherapy *in vivo*[18,19].

Type 1 intefereron production.

Recently, we identified yet another signal that contributes to the immunogenicity of cell death. We observed that cancer cells expressing the double-stranded RNA sensor TLR3 can produce type-1 interferons (IFN-1) after treatment with anthracyclines or oxaliplatin *in vitro* or *in vivo*. Apparently, IFN-1 then must act on its receptor (IFNAR) in an autocrine or

paracrine fashion to favor the anticancer immune response. Knockout of IFNAR in the immune system has no major inhibitory effect on the anticancer immune response elicited by dying tumor cells. However, knockout of IFNAR in cancer cells curtails their capacity to elicit such a response. Downstream of IFNAR, tumor cells produce the chemokine CXCR10, which then acts to attract T lymphocytes (and perhaps other immune cells) into the tumor bed[20].

Altogether, there results indicate the existence of four hallmarks of ICD, namely (i) CRT exposure, (ii) ATP release, (iii) HMGB1 exodus and (iv) IFN-1 secretion. Each of these hallmarks is essential for the immunogenicity of cell death in a non-redundant fashion (Figure 1). At present it appears plausible, yet remains to be explored, that these characteristics of ICD must be produced by several cancer cells located in close proximity to each other (rather than on single, isolated cells). This 'design' of the system might constitute a fail-safe mechanism that reduces the likelihood of immune reaction when apoptosis is a sporadic event.

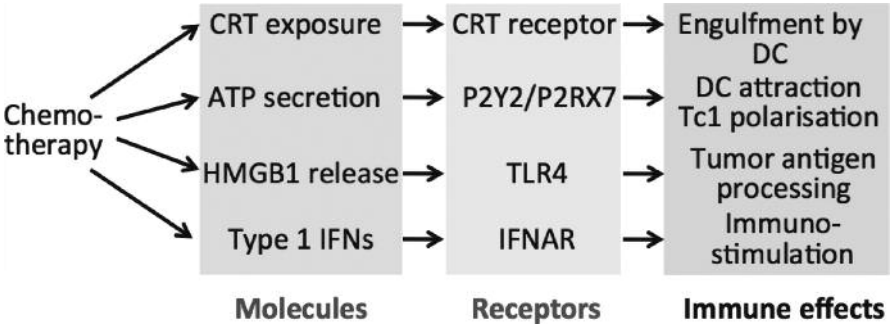

Figure 1 Central hypothesis of the present research project. Chemotherapy must induce (at least) four molecular species to be exposed on the surface of cancer cells or to appear in soluble form in their vicinity. These ligands then act on a series of specific receptors that ultimately stimulate the capacity of DCs to prime/activate cytotoxic T cells specific for tumor-associated cell antigens.

Our current working hypothesis suggests that the immune system dictates the long-term success of anti-cancer therapies, and that this immune response is determined by immunogenic tumor cell death (20,21). Thus, therapeutic failure can result from the incapacity of cancer cells to undergo ICD (rather than cell death as such). Only chemotherapeutics that elicit ICD may mediate a long-term therapeutic success. In addition, tumors that are unable to undergo ICD are intrinsically incurable. Similarly, genetically or acquired defects that reduce the capacity of the immune system to sense ICD-associated signals reduce the efficacy of anticancer chemotherapy with anthracyclines or

oxaliplatin[20,21]. We are currently developing therapeutic strategies that restore deficient ICD signals with the objective of ameliorating present and future chemotherapies.

In addition, we are currently screening large drug libraries for the identification of drugs that are able of inducing ICD using the established hallmarks of this cell death modality[23-25]. So far, we have found that approximately 5% of all currently used anticancer agents (6 out of 114) are endowed with the capacity of stimulating all hallmarks of ICD. In contrast, we found that among the mechanistic set of anticancer agents that is being developed by the National Cancer Institute (NCI) of the United States of America, only 0.1% of the drugs (1 out of 879) act as *bona fide* ICD inducers. This suggests that clinical development of successful anticancer agents has led to a selection in favor of components that are able to induce ICD, contrasting with the merely pre-clinical (and non-immunological) development that has yielded the collection of cytotoxicals assembled by the NCI. We surmise that prescreening of prospective anticancer agents for ICD induction might constitute a strategy for ameliorated selection of successful drugs.

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Specialty and Present Interest:
Apoptosis, Autophagy, Chemotherapy, Endoplasmic Reticulum Stress, Necrosis

ROLE OF IMMUNE-SUPPRESSIVE CELLS IN THE SURVIVAL OF GASTROINTESTINAL CANCER PATIENTS TREATED WITH STANDARD CHEMOTHERAPY

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Recent progress in cancer immunotherapy has been astonishing, and several immune checkpoint inhibitory antibodies have been able to induce remarkable therapeutic benefits against several cancers. Cancer immunotherapy is now securing a position as a standard therapy.

Thinking back to the history of cancer treatment, chemotherapy including molecular target agents has been a standard therapy against advanced cancers. Many oncologists have imagined that because chemotherapy damages the immune status of the patient, it is inconsistent with immunotherapy. However, in recent years, some anti-cancer drugs have been found to induce positive immune reactions against cancers, emerging a new concept of 'immunogenic chemotherapy'.

Several reports have suggested the responsiveness to first-line chemotherapy as a predictive marker of the responsiveness of second and subsequent chemotherapies, even though the mechanisms of each anti-cancer drug differ. Various factors, including type of gene mutation, epigenetic change, and genetic polymorphisms, have been reported to be associated with response to each drug; however, the reasons why the same patients respond to agents with different mechanisms remain unclear. So, we examined what immunological factors affect the response and survival of cancer patients treated with standard chemotherapeutic regimens.

In our study, we prospectively enrolled patients with colorectal, gastric, and esophageal cancers who were due to undergo standard chemotherapies at the National Cancer Center Hospital from June 2013 to present. After obtaining written informed consent, we collected peripheral blood before and under treatment, and at 6 months after initiation of

chemotherapy. We evaluated myeloid-derived suppressor cells, T cell subsets including regulatory T cells and natural killer cells using flow cytometry.

Our study showed that the high frequency of immune-suppressive cells such as MDSCs were strongly associated with poor survival even in patients with colorectal and gastric cancer, who received the standard chemotherapeutic regimens. Tumor-infiltrating immune cells represent a local immunological status. Our study also showed that tumor-infiltrating myeloid cells were also involved in prognosis of patients with cancers.

Furthermore, the depletion of MDSC significantly suppressed the tumor growth and prolonged survivals in murine cancer models treated with chemotherapeutic reagents. Therefore, the immune-suppressive cells may be a novel promising target in gastrointestinal cancer patients treated with standard chemotherapies.

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IMMUNE CHECKPOINT BLOCKADE IN CANCER THERAPY: NEW INSIGHTS AND OPPORTUNITIES

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The existence of multiple non-redundant l inhibitory pathways that limit T cell responses offers novel strategies for mobilizing the immune system to attack cancer cells. The best characterized of these immune checkpoints is CTLA-4, which inhibits CD28 mediated costimulation. Antibodies to CTLA-4 have proven effective against multiple tumor types in both pre-clinical and clinical studies. Ipilimumab, an antibody to human CTLA-4, showed long term (>4.5 years) survival benefit in about 23% of patients in a randomized, placebo-controlled trial in late stage melanoma. In 2011 it was approved by the FDA for treatment of late stage melanoma and is now a standard of care for that disease. A recent retrospective study of almost 5,000 patients showed an inflection point at about 2.5 years with essentially no deaths of about 20% of patients for 10 years following treatment.

The mechanism(s) of action of anti-CTLA-4 are still being elucidated. We and others have shown that CLTA-4 limits T cell proliferation by a cell intrinsic mechanism. However, there is also evidence that anti-CTLA-4 has to engage the target on both effector (Teff) and regulatory (Treg) T cells. We have recently uncovered a mechanism whereby anti-CTLA-4 antibodies expand Treg in lymph nodes but cause their depletion in the tumor microenvironment. Thus anti-CTLA-4 exerts its anti-tumor effects by multiple mechanisms. We have also shown that CTLA-4 blockade results in a 2-5 fold increase in the frequency of CD4 T cells expression ICOS (inducible costimulator) in both tumor tissues and blood. This population contains that vast majority of tumor antigen specific cells that produce IFNγ and TNFα. The appearance of the ICOS+ CD4 cells serves as a pharmacodynamic marker of a biological effect of anti-CTLA-4 activity. Using mouse

models, we have shown that the ICOS/ICOSL pathway is critical for optimal anti-tumor activity of anti-CTLA-4. Furthermore, we have shown that agonist stimulation of ICOS coupled with CTLA-4 blockade results in enhanced anti-tumor efficacy in mouse models, suggest that ICOS is a compelling molecule to develop as a target for agonistic targeting of costimulatory checkpoints.

PD-1, another checkpoint, recruits a phosphatase and seems to interfere with T cell antigen receptor mediated signaling. It has two ligands, PD-L1 and PD-L2, which are both expressed on dendritic cells. However, many tumor cells also express PD-L1. Antibodies to PD-1 and PD-L1 have both shown objective responses against several tumor types in clinical trials with response rates of about 25% . A recent phase II trial of a combination of anti-PD-1 and anti-CTLA-4 in melanoma showed objective responses in about 50% of late stage melanoma patients. Our studies indicate that the mechanisms of anti-PD-1 mediated tumor immunity are distinct from those of anti-CTLA-4, at least as for the role of ICOS+ CD4 T cells.

These studies and their implications for cancer therapy are discussed.

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1984-2004	Professor, Division of Immunology Director, Cancer Research Laboratory Univ. of California, Berkeley
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THERAPEUTIC MODULATION OF THE IMMUNE MICROENVIRONMENT OF CANCER

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Major advances have been made in the immune-based therapy of cancer by antibody blockade of immune inhibitory pathways such as CTLA-4 and PD-1. Anti-PD-1 antibodies have produced objective responses in one third to one half of patients with advanced, chemotherapy refractory melanoma and renal cancer and one quarter of patients with non-small cell lung cancer. Recently, significant response rates to anti-PD-1 antibodies have been shown in bladder cancer, head and neck cancer, gastroesophageal cancer, ovarian cancer and lymphoma. These responses are highly durable, the majority lasting significantly greater than one year and beyond cessation of therapy. Further, expression by tumor cells of ligands for PD-1 is associated with higher response to anti-PD-1 therapy. In exploring the basis for up-regulation of the major PD-1 ligand, PD-L1, on tumor cells, we found that its expression is not constitutive, but rather, is highly associated with lymphocytic infiltration. We identified IFN-γ as an immune signal sensed by the tumor cell that induces PD-L1 expression. In addition to IFN-γ, genes associated with Th1 responses, CTL responses and other inhibitory molecules, such as LAG-3, are up-regulated in lymphocytic infiltrates associated with PD-L1^{+} tumor cells. These findings indicate that multiple counterbalancing immune effector and inhibitory pathways are operative in the immune microenvironment. They led us to hypothesize a new mechanism of PD-L1 expression in tumors, termed adaptive resistance, distinct from a constitutive mechanism of PD-L1 expression in tumors. The adaptive resistance mechanism implies that other therapies such as vaccines may induce antitumor responses that in turn induce up-regulation of PD-L1. In such a circumstance, vaccination and PD-L1 blockade might produce synergistic anti-tumor activity. Using novel vaccine formulations, we indeed

demonstrate IFN-γ dependent induction of PD-L1 on tumors and synergistic activity such that the vaccine/anti-PD-1 combination.

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IMMUNOTHERAPY THAT TARGETS THE TUMOR AND THE HOST

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Cancer therapies can target the tumor or the host microenvironment, and especially the host immune system. An exciting challenge is to combine these therapies in ways that are synergistic.

Improving the power of monoclonal antibodies

The therapeutic power of antibodies can be enhanced by stimulating antibody dependent cellular cytotoxicity (ADCC), a primary mechanism of anti-tumor effect. One way to accomplish this is to target CD137, an activation molecule that appears on host NK cells after they encounter tumor cells coated by an anti-tumor antibody[1-3]. CD137 is also expressed on activated T cells and therefore the antibody against CD137 might also enhance an underlying host anti tumor T cell immune response. Clinical trials are now under way to test the effect of an antibody against CD137 as a single agent and in combination with Rituximab in lymphoma, with Traztuzumab in breast cancer and with Cetuximab in colon cancer.

In situ therapeutic vaccination

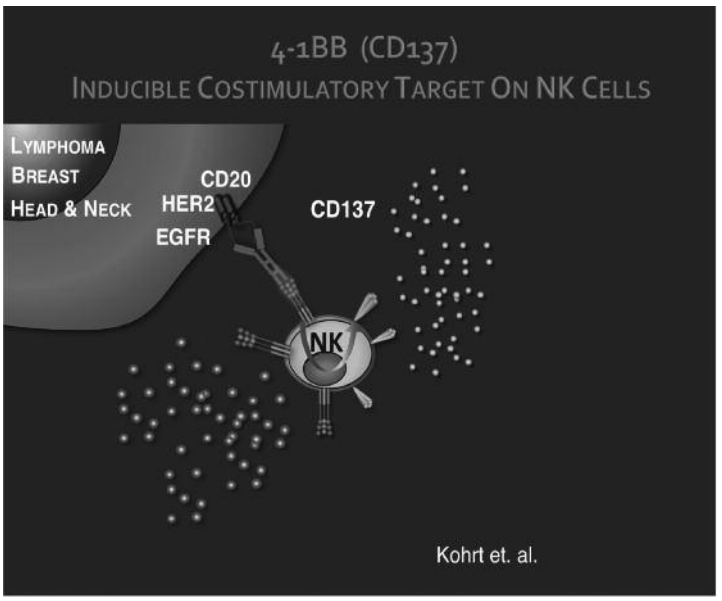
Abscopal tumor responses can be induced in patients with lymphoma by local *in situ* vaccination. CpG oligonucleotide, a ligand for a Toll-like receptor, is injected into one site of tumor and low dose radiation is directed to the same site[4-6].

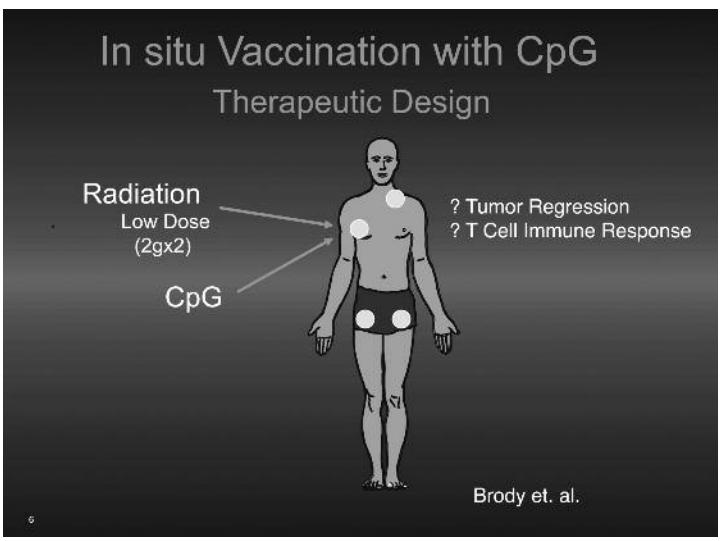
In animal models this treatment generates an anti-tumor T cell immune response. These T cell responses can be enhanced by intra-tumoral injections of antibodies against CTLA4 and OX40[7]. These antibodies work by depleting the negative T regulatory cells (Tregs) in

the local tumor microenvironment, unleasing T effector cells to attack tumor at distant sites. Very low doses of checkpoint antibodies can be used to avoid autoimmunity. Such combinations of low dose radiation with immune enhancing agents may change the way we employ radiotherapy in the future- to help trigger immune responses rather than to sterilize the local tumor site.

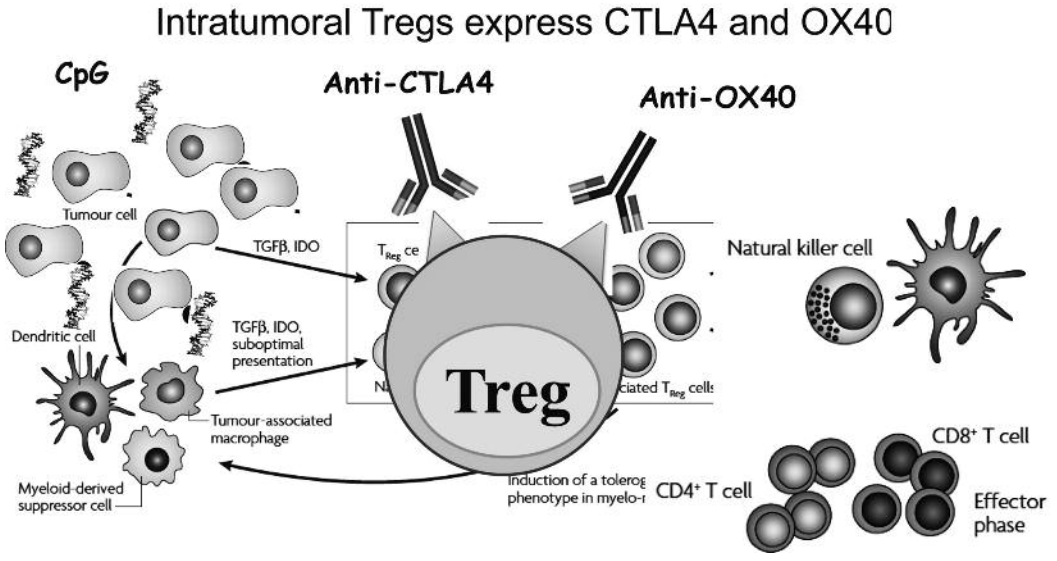
Combination of immunotherapies with small molecule-targeted therapies

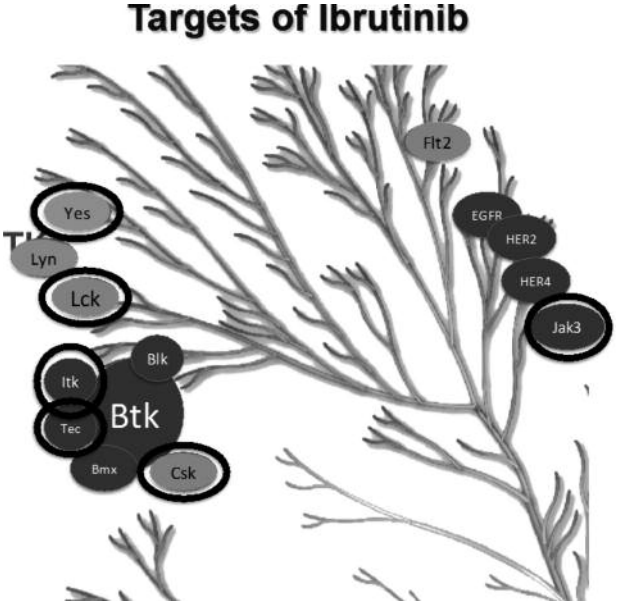
Small molecule inhibitors of survival pathways in lymphoma cells, such as B cell receptor signaling, also have effects on the immune system. A case is point is Ibrutinib, a





covalent inhibitor of Bruton's tyrosine kinase (Btk), recently approved for the treatment of CLL and MCL and with significant clinical activity in other lymphoid malignancies. Ibrutinib inhibits other members of the Tec kinase family, such as Itk, an important signaling molecule in subsets of T cells. By doing so, it can sculpt the T cell immune response and direct it against the tumor. Recent preclinical experiments indicate that Ibrutinib can enhance the T cell immune response induced by *in situ* vaccination as well as other immunotherapeutic maneuvers.





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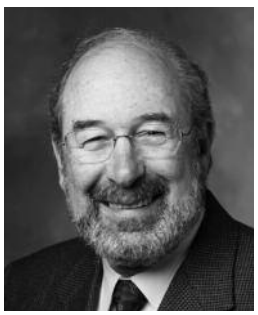

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COMBINATION CHECKPOINT BLOCKADE AND THE ROLE OF 'PASSENGER' MUTATIONS IN CLINICAL RESPONSE TO IPILIMUMAB

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The past decade has been a period of significant progress in the area of cancer immunotherapy, enabled by significant advances in basic science allowing for a better understanding of molecular mechanisms of immune regulation and immune cell dynamics. These successes reflect a close collaboration between basic, translational and clinical scientists.

Blockade of cytotoxic T lymphocyte antigen 4 (CTLA-4) was suggested to be an impactful means to augment tumor immunity based on the pioneering studies of Jim Allison, who hypothesized that impairment of this critical molecular brake would overcome tumor-induced immune suppression and allow for more robust immune response to tumors.[1] In 2011, the US FDA approved ipilimumab for the treatment of metastatic melanoma with two randomized phase 3 trials demonstrating prolongation of overall survival, the first intervention ever to demonstrate a survival benefit in patients with metastatic melanoma.[2,3] Investigation of blockade of the programmed death-1 (PD-1) pathway followed very shortly thereafter. PD-1 is a marker on T cells which, while being up-regulated after activation, serves to mark T cells as being exhausted. When engaging one of its ligands (PD-L1 or PD-L2), PD-1 can lower the threshold for apoptosis in T cells.[4,5] A growing number of antibodies that block either PD-1 or PD-L1 have entered clinical trials with some recently approved (nivolumab, pembrolizumab) for metastatic melanoma and others currently in later stage investigation for additional cancers. It was not completely surprising that PD-1 blockade showed clinical efficacy in melanoma and renal cell carcinoma. However, Julie Brahmer and Suzanne Topalian at Johns Hopkins observed that patients with non-small cell lung cancer could respond to PD-1 blockade with nivolumab,

forever changing that notion that melanoma and renal cell carcinoma were the only solid tumors amenable to immune modulation.[6,7] The list of malignancies where PD-1 pathway blockade has been demonstrated significant activity also now includes head and neck squamous cell carcinoma, urothelial bladder cancer, ovarian cancer, gastric cancer and both Hodgkin's and non-Hodgkin's lymphomas.

Given the activity noted with both CTLA-4 and PD-1 blockade and the complementary mechanisms of action as well as supportive pre-clinical data, clinical trials are now investigating combination checkpoint blockade. The most mature data has come from a Phase 1 study combining ipilimumab and nivolumab in patients with melanoma which showed an overall response rate of 40% across dose cohorts with over 50% of patients in some cohorts showing rapid and deep (>80% reduction) tumor regressions (Figure 1).[8] These responses were seen in the context of manageable mechanism-based toxicity, which had previously been observed in trials of CTLA-4 and PD-1 pathway blockade as monotherapies. Patients treated with combined checkpoint blockade tend to have rapid and deep responses, materially distinct from those observed in monotherapy trials. Such responses are generally durable, even in some cases when treatment was stopped early due to toxicity. Unlike in studies of PD-1 blockade as monotherapy, there was no significant difference in clinical activity based on tumor expression of PD-L1, suggesting the inflammation mediated by ipilimumab could enhance PD-L1 or other relevant marker

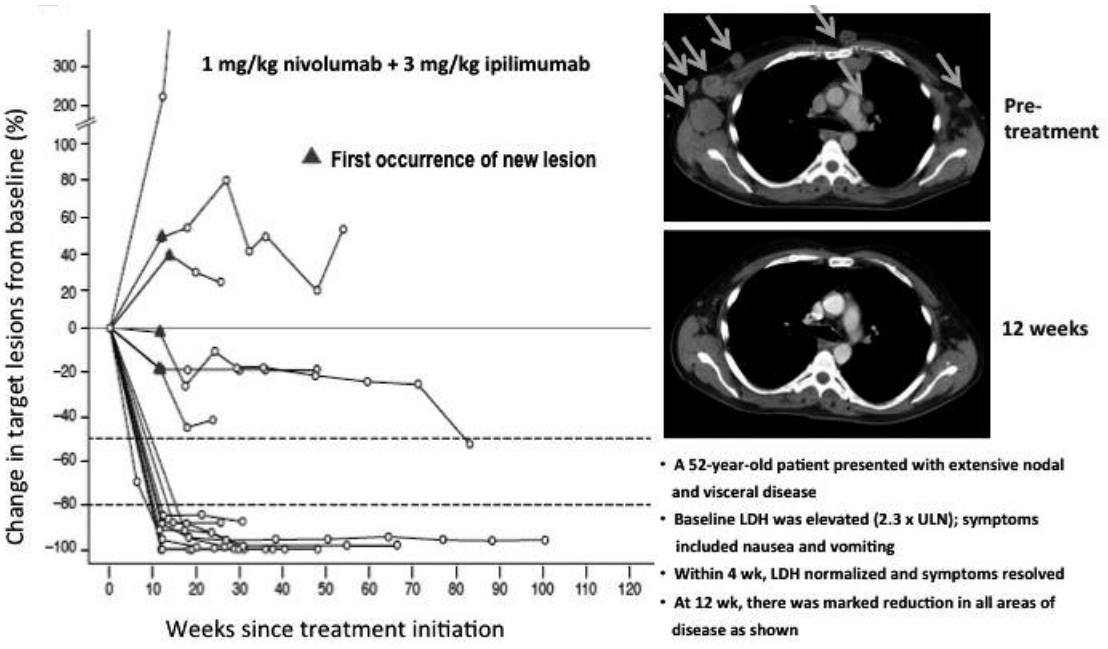

Figure 1 Rapid and Durable Changes in Target Lesions

expression that sensitizes to PD-1 blockade (Figure 2). However, this remains a hypothetical explanation that requires mechanistic confirmation.

Early studies of the immune dynamic correlates in patients treated with the combination therapy show a high spike in proliferating $CD4^+$ and $CD8^+$ T cells occurring within days after the first dose. This was not evident in patients treated with nivolumab after receiving prior ipilimumab. Pharmacokinetic analysis of patients treated sequentially with ipilimumab followed by nivolumab suggest that the plasma concentration of ipilimumab remaining when nivolumab is initiated could be important as a higher response rate was noted for patients with higher than the mean concentration. Phase 2 and 3 trials of this combination are ongoing in melanoma with phase 1 programs in numerous other tumor types. Similar strategies are being investigated using a combination of other CTLA-4 blocking antibodies such as tremelimumab and other inhibitors of the PD-1 pathway such as the anti-PD-L1 antibody, MEDI-4736. The above approaches are also being considered as combinatorial partners both with other immunotherapies as well as with treatments that directly affect the cancer cell.

Recently, attention is being paid to the mechanisms underlying the clinical efficacy of checkpoint blockade in certain malignancies. One hypothesis has been that cancers that have a high mutational load may be more amenable to immune modulation by virtue of the larger number of potential neo-epitopes present, fostering baseline immune recognition

Cohort [n]	Evaluable Samples	ORR, n (%)	
PD-L1 Status		PD-L1+	PD-L1-
Concurrent Cohorts 1-3 [53]	36	8/14 (57)	9/22 (35)
Cohort 8 [41; Nivo1 + IPI3]	20	0/0	8/20 (40)
Sequenced [33]	23	5/8 (63)	3/15 (20)

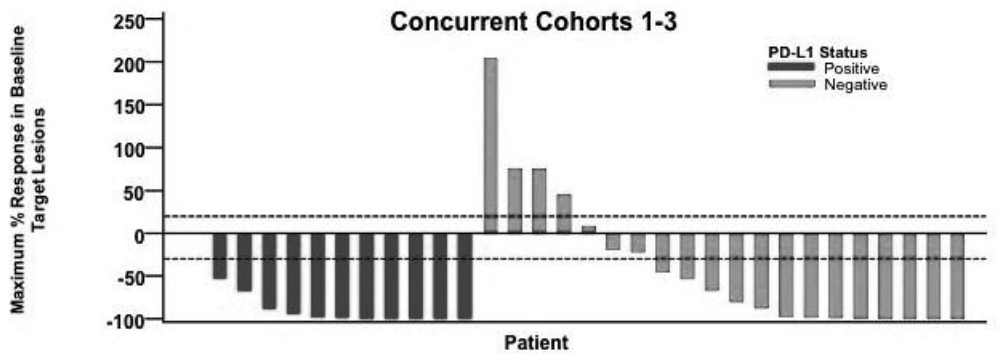

Figure 2 Response Rate by PD-L1 Status (5% cutoff)

that can then be potentiated by checkpoint blockade. This is supported by referring to the spectrum of mutational loads across cancer, in which the majority of the malignancies that have been shown to respond to these immunotherapeutic strategies have a higher number of mutations.[9] We have recently found that metastatic melanoma patients having long term clinical activity after ipilimumab treatment have a significantly greater median number of non-synonymous passenger mutations, compared with patients who do not respond or those who have only short-term (< 6 months) regression (Figure 3).[10]

The use of whole exome sequencing has allowed us to explore the additional hypothesis that the higher likelihood of durable response and long-term survival observed may not simply be explained by a higher quantity of mutations but rather that the individual immunologic quality of mutations is also important. This could serve to explain the presence of outliers in the prior analysis, where some patients with low mutation burden had long-term survival while others with quite higher numbers of mutations (1000-1500) did not respond to ipilimumab treatment. Using a novel bioinformatics platform, we have found that there are substrings of class I epitopes (tetrapeptides) which are shared by the clinical responders and not found in the non-responders. Intriguingly, the vast majority of these favorable substrings are also found in known epitopes derived from proteins found in bacteria and viruses. This suggests the biologically interesting hypothesis that a high

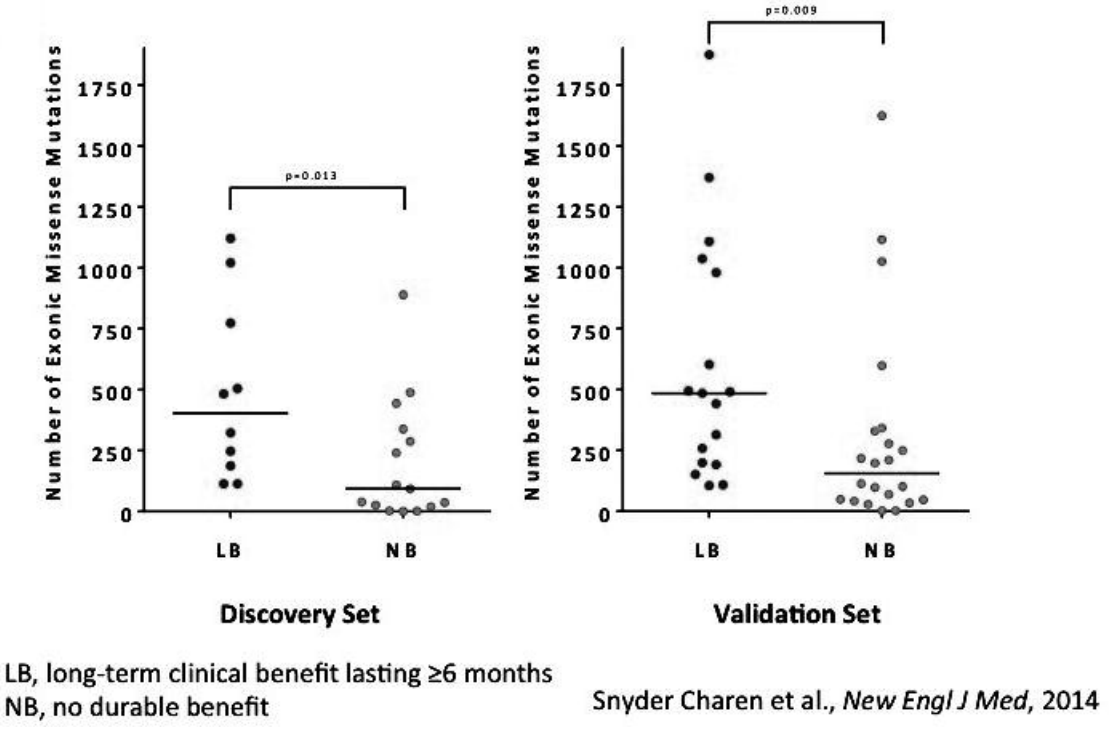

Figure 3 Mutational Load Correlates with Clinical Outcome

mutational load can lead to a greater probability of a tumor-derived peptides appearing structurally similar to truly foreign antigens.

The confluence of advances in multiple areas of cancer research have allowed for the development of new and effective strategies to durably control cancer. Our task now is further understanding the mechanistic basis of such activity and to leverage the recent knowledge gained in the area of mutational neo-epitopes to devise strategies to initiate tumor immunity in patients who are not currently responding and to predict responses before treatment is initiated.

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THERAPY-INDUCED ANTIBODY RESPONSES

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Detailed histopathologic analysis of resected tumors in large cohorts of clinically annotated patients has established the prognostic importance of intra-tumoral immune infiltrates[1-3]. A coordinated humoral and cellular response with associated tertiary lymphoid structures is tightly correlated with prolonged survival after standard oncologic therapy[4]. A systems biology approach has linked $CD8^+$ cytotoxic T lymphocytes, a Th1 cytokine signature, $CD4^+$ T follicular helper cells, and B cells to favorable outcomes[5]. While these endogenous reactions develop in only a small subset of patients, their association with durable disease control suggests that a major goal for active immunotherapy should be to enhance the proportion of patients who mount this type of immune response.

Vaccination with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) engenders a coordinated cellular and humoral anti-tumor responses in some patients with solid or hematologic malignancies[6]. Examination of metastases resected after vaccination, but not before therapy, reveals the induction of brisk $CD4^+$ and $CD8^+$ T cell and $CD20^+$ B cell infiltrates associated with tumor destruction[7]. A tumor-specific vasculopathy with lymphoid and granulocytes disrupting small tumor-blood vessels in association with zonal areas of ischemic necrosis is also observed[7]. The subsequent administration of fully human monoclonal antibodies that block the negative T cell costimulatory molecule cytotoxic T lymphocyte associated antigen-4 (CTLA-4) intensifies these therapeutic effects, resulting in long-term survival in the absence of serious inflammatory pathology[8]. Pathologic analysis of metastases obtained after the sequential vaccination/immune checkpoint blockade revealed a linear relationship between the extent of tumor necrosis and the natural logarithm of the ratio of

infiltrating $CD8^+$ cytotoxic T cells and $FoxP3^+$ Tregs. Moreover, the tumor-specific vasculopathy was again observed. Together, these results suggest that identification of strategies to enhance the proportion of patients that generate high $CD8^+$ cytotoxic T cell to $FoxP3^+$ Treg ratios and responses that target the tumor vasculature should be a high priority.

The generation and analysis of GM-CSF deficient mice uncovered an unexpected dual role for the cytokine in tumor immunity[9]. The development of chronic inflammatory disease in the lung and susceptibility to colitis revealed a key role for GM-CSF in immune tolerance[10,11]. This pathway involves the ability of GM-CSF to promote the efficient phagocytosis of apoptotic cells by dendritic cells and macrophages through the upregulation of phosphatidylserine binding proteins such as milk fat globule epidermal growth factor-8 (MFG-E8); binding of MFG-E8 to cognate $\alpha_v\beta_{3/5}$ integrins on phagocytes in turn triggers upregulation of Twist, TGF-β, and CCL22, which are critical for the homeostasis of $FoxP3^+$ Tregs[9]. GM-CSF deficiency thus results in attenuated Treg responses that likely contribute to the inflammatory pathology. Notwithstanding this tolerance mechanism, GM-CSF also contributes to the induction of protective T and B effector responses when a second activating signal is provided, such as concurrent engagement of toll-like receptors. In this setting, stimulation through diverse TLRs downregulates the tolerance pathway and instead switches GM-CSF function towards dendritic cell maturation and tumor protection.

A recent clinical trial combining systemic GM-CSF protein and anti-CTLA-4 antibodies (ipilimumab) in advanced melanoma patients supports the concept of a dual role for GM-CSF in tumor immunity[12]. In the study, 245 patients with unresectable stage III or IV metastatic melanoma were randomized to ipilimumab alone or combined with GM-CSF. At a median follow-up of 13.3 months, patients that received GM-CSF and ipilimumab showed a reduced incidence of serious gastrointestinal and pulmonary pathology, consistent with the murine studies that delineated a key role for the cytokine in maintaining immune homeostasis at mucosal interfaces. GM-CSF also improved the one-year survival, increasing from the 52.9% with ipilimumab alone to 68.9% for combined GM-CSF and ipilimumab. Although the precise mechanism for the therapeutic advantage remains to be clarified, one interesting possibility might involve the ability of GM-CSF to enhance anti-CTLA-4 mAb mediated antibody dependent cellular cytotoxicity (ADCC) through tumor infiltrating myeloid cells. Studies in murine models established a key requirement for Fc receptors and ADCC in the therapeutic effects of anti-CTLA-4 mAbs, likely because tumor-infiltrating Tregs express the highest levels of CTLA-4 of any cell in a tumor-bearing host[13]. The selective reduction of Tregs in the tumor microenvironment might allow GM-CSF function to be skewed more toward protective tumor immunity.

The delineation of a dual role for GM-CSF in tumor immunity suggests novel strategies to enhance the overall therapeutic effects of the cytokine. In one approach, a dominant negative inhibitory form of MFG-E8 that retains the ability to bind phosphatidylerine but is impaired in signaling through $\alpha_v\beta_{3/5}$ integrins (MFG-E8-RGE) could be used to block the tolerance pathway[14]. Pre-clinical studies in several murine models demonstrated that vaccination with irradiated tumor cells engineered to co-express GM-CSF and MFG-E8-RGE stimulates higher levels of tumor immunity compared to GM-CSF or MFG-E8-RGE alone[9]. The improved tumor protection involves the generation of a higher $CD8^+$ T effector to $FoxP3^+$ Treg ratio and a higher titer of anti-tumor antibodies (Figure 1). To translate this therapeutic concept to "first-into-human" testing in patients, we have generated human K562 cell lines that stably express human GM-CSF and the human MFG-E8-RGE mutant. Studies that use peripheral blood mononuclear cells from healthy donors have revealed that the human MFG-E8-RGE mutant attenuates the tolerance functions of human GM-CSF. Based on these results, we plan to submit an Investigator Sponsored New Drug Application to the FDA to advance this vaccination strategy to patient testing.

In a second approach, material science engineering has been applied to develop a more potent cellular vaccine strategy based on the dual functions of GM-CSF. Porous, poly-(lactide-coglycolide) scaffolds have been generated that provide precise spatial and temporal control over the release of encapsulated agents[15-17]. The polymers have been

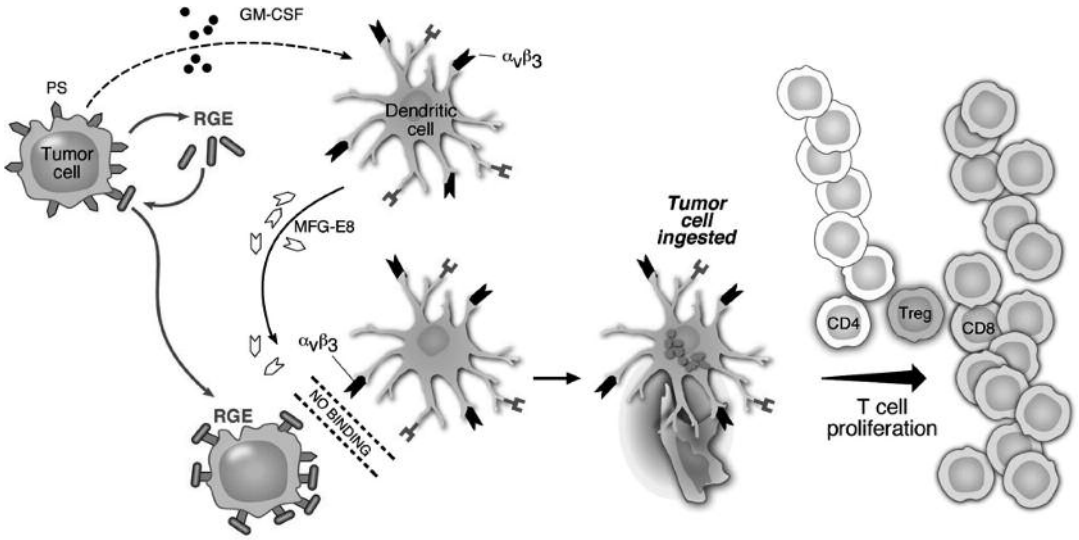

Figure 1 Schema of combined GM-CSF/MFG-E8-RGE secreting tumor cell vaccines. The dominant negative inhibitor MFG-E8-RGE binds phosphatidylserine present in the vaccine microenvironment, but is unable to signal through $\alpha_v\beta_{3/5}$ integrins to promote Treg responses. A higher ratio of $CD8^+$ Teffectors to $FoxP3^+$ Tregs is generating, enhancing anti-tumor immunity. Reprinted from (6).

extensively used in the context of resorbable sutures and manifest a very favorable safety profile. The inclusion of recombinant GM-CSF protein, TLR ligands (including agonists of TLR3, 4, or 9), and necrotic tumor cell lysate in the scaffolds results in a potent and sustained local immune response following implantation in subcutaneous pockets in immunocompetent mice (Figure 2). This combination of agents results in a broad dendritic cell response that includes $CD8^+$ and $CD11b^+$ classical dendritic cell subsets and plasmacytoid dendritic cells. The cross-talk among these subsets leads to the production of high levels of Th1 cytokines but the attenuation of immunoregulatory cytokines, engendering a high ratio of $CD8^+$ T effectors to $FoxP3^+$ Tregs that accomplish the rejection of established tumors. A "first-into-human" trial of this material scaffold vaccine has been initiated in advanced melanoma patients.

In addition to enhancing T cell immunity, GM-CSF based vaccines elicit a broad humoral reaction directed towards intracellular, surface, and secreted tumor proteins[18]. Through screening tumor derived cDNA expression libraries and protein arrays with sera from patients who achieved long-term clinical responses to autologous GM-CSF secreting tumor cell vaccines and CTLA-4 blockade, we identified multiple targets of high titer antibodies. A subset of these likely plays critical roles in tumor pathogenesis. For example, antibodies directed against major histocompatibility complex chain-related protein A (MICA) antagonize the immunosuppressive effects of soluble MICA, thereby promoting innate and adaptive anti-tumor cytotoxicity[19]. Moreover, antibodies to angiogeneic cytokines including vascular endothelial growth factor-A (VEGF-A), angiopoietin-1/2, and macrophage migration inhibitory factor likely contribute to the tumor-selective vasculopathy through blocking the angiogeneic activities of these factors[20]. Methods to

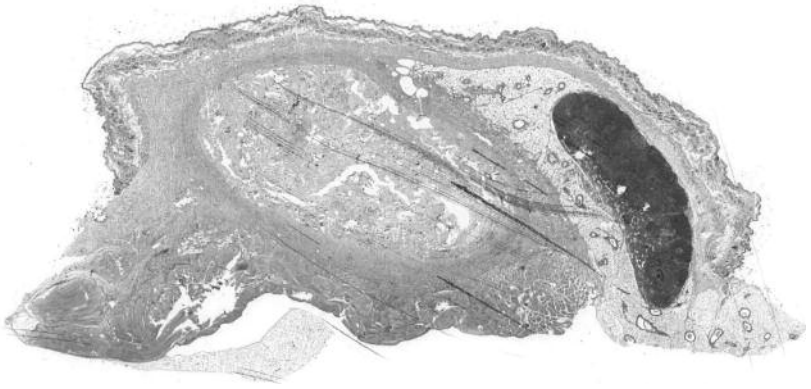

Figure 2 Material engineered scaffold vaccines elicit potent vaccine responses. Porous, PLGA scaffolds incorporating recombinant GM-CSF protein, CpG oligonucleotides, and necrotic B16 cell lysates were implanted into a subcutaneous pocket of a C57Bl/6 mouse. A brisk infiltrate of dendritic cells, macrophages, granulocytes, T and B cells, and stromal elements is evident.

isolate the rare memory B cells that produce these functionally relevant antibodies have been developed[21]. These isolated monoclonal antibodies provide important insights into the mechanisms through which humoral immunity contributes to tumor destruction and are attractive candidates for clinical development as novel immunotherapies.

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CLINICAL APPLICATION OF ANTI-CCR4 MONOCLONAL ANTIBODY

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Immunotherapy offers an attractive approach to treat cancer by specifically targeting tumor cells and by modulating the host immune reactions. A number of antibodies have recently been developed and used for the treatment of malignant diseases. Here, we present a review of our research strategies for developing novel therapeutics using a humanized monoclonal antibody for treatment of hematological malignancies and cancers.

Clinical application for Lymphoid Neoplasms

Research to develop the anti-CCR4 monoclonal antibody (Mogamulizumab) as an anticancer antibody drug for adult T-cell leukemia-lymphoma (ATL) started in 1999, with a company that successfully produced a mouse monoclonal antibody (KM2160) against a chemokine receptor, CCR4. The target molecule CCR4 is a 7 transmembrane G protein coupled receptor (GPCR), which recognizes two functional ligands, TARC (thymus and activation-regulated chemokine; CCL17) and MDC (macrophage-derived chemokine; CCL22). It is expressed mainly by Th2 cells and $FOXP3^+$ T regulatory (Treg) cells among normal lymphocytes. CCR4 is expressed on most ATL cells[1], and a subgroup of peripheral T-cell lymphomas (PTCL)[2].

ATL is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type 1. The disease is resistant to conventional chemotherapy, and there currently exist very limited treatment options; thus, it has a poor prognosis, with a median progression-free survival (PFS) and overall survival (OS) of 7.0 and 12.0 months, respectively, when using the best combination chemotherapy in previously untreated ATL patients. Therefore, the development of alternative treatment strategies for patients with

ATL is an urgent issue.

Mogamulizumab is a first-in-class defucosylated humanized anti-CCR4 monoclonal antibody, which is engineered to exert higher antibody-dependent cell-mediated cytotoxicity (ADCC) activity using a defucosylation technology we named Potelligent technology, developed by the Kyowa Hakko Kirin Co. Ltd. The ADCC activity of Mogamulizumab has a 2–3 log stronger activity than conventional antibodies. Furthermore, Mogamulizumab has no complement dependent cytotoxicity (CDC) activity, no neutralizing activity and does not directly induce apoptosis[3]. Since this agent showed potent antitumor activities in patients with relapsed ATL (NCT00355472, NCT00920790)[4,5] or PTCL and cutaneous T-cell lymphoma (CTCL) (NCT01192984)[6], it was approved for the treatment of relapsed/refractory ATL in 2012 as an orphan drug, and for PTCL in 2014, in Japan. (Figure 1). Excellent treatment results have been reported in line with previous studies[3-6]. For example, Mogamulizumab monotherapy demonstrated significant responses in patients with relapsed ATL with an acceptable toxicity profile. An overall response rate of 50% and median PFS and OS values of 5.2 and 13.7 of months, respectively, were observed. The most common adverse events were infusion reactions (89%) and skin rashes (63%), which were manageable and reversible in all cases. It was also reported that objective responses were noted for 13 of 37 patients (35%; 95% confidence interval [CI], 20% to 53%), including five patients (14%) with complete responses in a multicenter phase II study for patients with relapsed PTCL and CTCL.

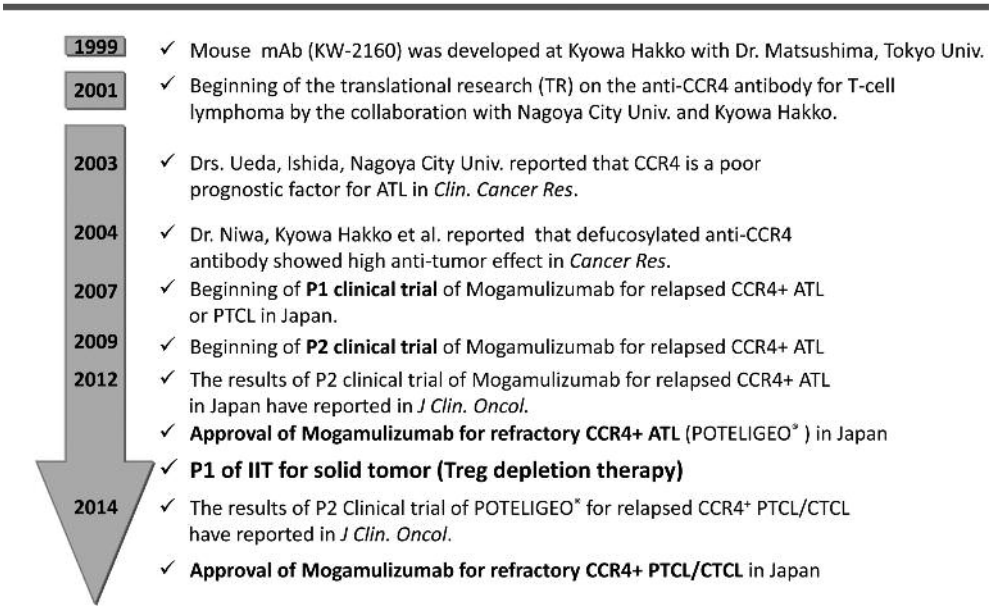

Figure 1 History of Translational Research of anti-CCR4 mAb (Mogamulizumab)

Feasibility of anti-CCR4 monoclonal antibody towards cancer immunotherapy

CCR4 is expressed on $CD45RA^{-}FOXP3^{high}CD4^{+}$ Treg cells [effector Treg (eTreg) cells], but is not expressed on $CD45RA^{+}FOXP3^{high}CD4^{+}$ Treg cells [naïve Treg (nTreg) cells][7], therefore, anti-CCR4 monoclonal antibodies deplete only eTreg cells but not nTreg cells[8]. Tumor sites were shown to be intensively infiltrated with $FOXP3^{+}CCR4^{+}$ eTreg cells[8,9], which can be depleted by anti-CCR4 monoclonal antibody treatment[8]. In addition, the depletion of eTreg cells by anti-CCR4 monoclonal antibodies effectively augmented anti-tumor immunity compared with depletion by anti-CD25 monoclonal antibodies in an *in vitro* study[8]. Furthermore, in some ATL patients, *in vivo* administration of Mogamulizumab markedly reduced the eTreg-cell fraction and augmented anti-tumor immunity resulting in long lasting objective responses[5,10].

These results and observations indicate that CCR4 is an attractive target for cancer therapy, because eTreg cells play an important role in tumor escape from host immunity in the tumor microenvironment. Based on this concept, we are now conducting an investigator initiated phase Ia/Ib trial of Mogamulizumab for CCR4-negative advanced or recurrent solid tumors, specifically aiming to deplete eTreg cells and boosting antitumor immunity (NCT01929486) (Figures 2, 3).

It has been observed in phase Ia trial that KW-0761 is tolerated at dose range between 0.1 mg/kg to 1.0 mg/kg, demonstrating effective elimination of eTreg population in peripheral blood. Phase Ib trials currently underway.

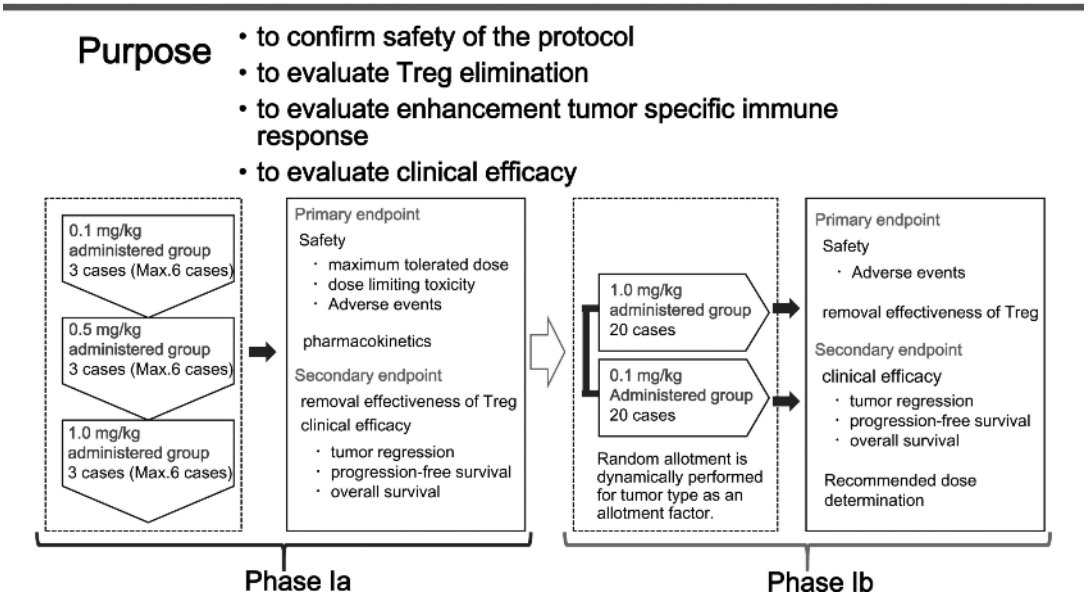

Figure 2 Monotherapy of Mogamulizumab for solid tumor by investigator initiated phase Ia/Ib trials
Chief Investigator: R Ueda
Co-investigators: E Nakayama, M Oka, H Udono, Y Doki, H Wada, H Nishikawa, S Iida, T Ishida, H Inagaki, T Doki, K Kakimi, K Funakoshi, E Sato

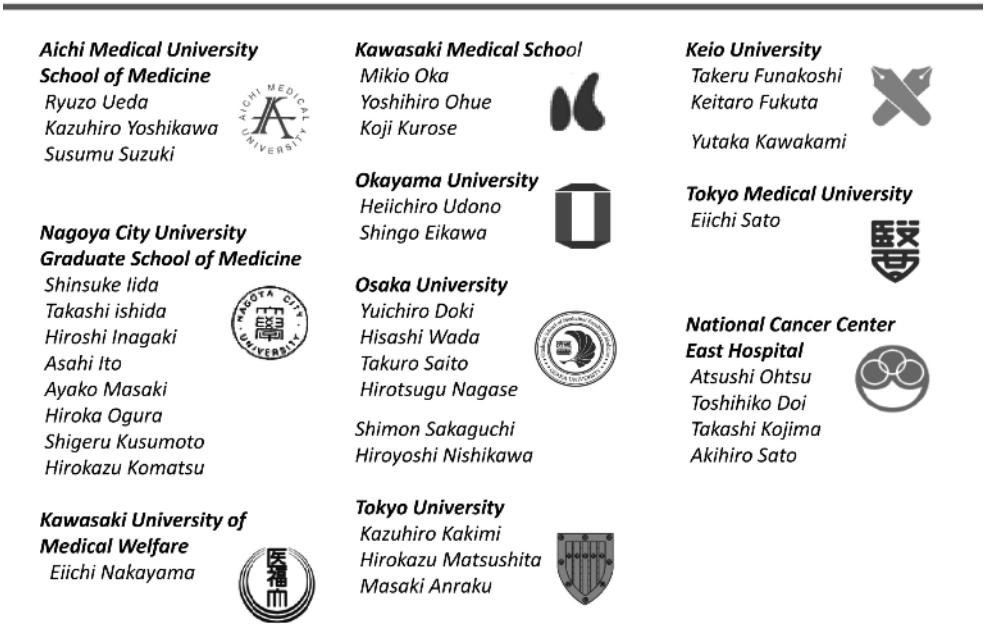

Figure 3 Collaboration members of Treg depletion project

On the other hand, alterations of Treg-cell distribution or frequencies may contribute to various types of autoimmune diseases. In ATL patients who were administered Mogamulizumab, skin rash was frequently observed[11]. It is therefore a matter of some urgency to establish the safest and most effective treatment strategies for using Mogamulizumab in cancer patients[12]. In the near future, a new cancer immunotherapy combined with check point inhibitors and depletion of Treg cells with Mogamulizumab will be offered to appropriate cancer patients (Figure 4).

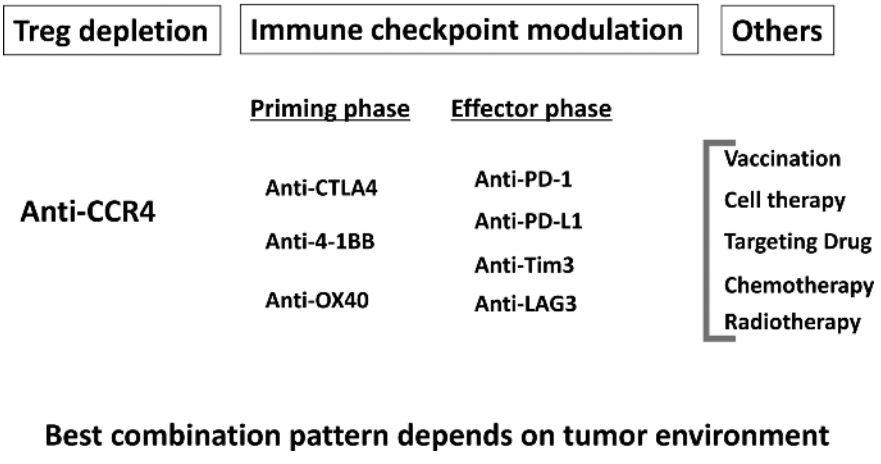

Figure 4 CCR4 immunotherapy in combination with immune checkpoint blockade

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Specialty and Present Interest:
Hematology/Oncology and Tumor Immunology
Molecular Target for Cancer, Monoclonal Antibody Therapy

DRUGGING THE UNDRUGGABLE WITH MONOCLONAL ANTIBODIES

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Most small molecule drugs currently available are directed to less than 5% of the proteins in the cell, typically those that are enzymes and therefore "druggable." Monoclonal antibodies (mAb) may be useful for a small number of proteins, all outside of the cell, which are not druggable by small molecules. However, the vast majoarity of proteins, particularly the important oncogenic proteins and mutated proteins, are inside of the cell and not accessible to mAb. Here we describe an approach to using mAb to target an important intracellular protein in cancer. The Wilms' tumor oncogene protein (WT1) is one such intracellular, oncogenic transcription factor that is over-expressed in a wide range of human cancers. Wt1 is a validated target for T cell-based immunotherapy, used in multiple clinical trials of vaccines and cellular therapies. RMFPNAPYL, "RMF", is a WT1-derived CD8 T cell epitope presented by HLA-A0201 on the surface of cancer cells.

We hypothesized that a "TCR-like" antibody or "TCR mimic" antibody (TCRm) specific for the WT1 peptide/HLA-A0201 complex might be an effective therapeutic agent. (Figure 1) We generated a high avidity (KD= 0.1 nM), fully human monoclonal antibody IgG1 (mAb), ESK1, specific for the WT1 RMF peptide/HLA-A0201 complex, using phage display technology[1-3]. ESK1 binds cancer and leukemia cell lines as well as primary cancer leukemia cells, which are both $WT1^{+}$ and $HLA\text{-}A0201^{+}$, as shown by flow cytometry and radioimmunoassays. ESK1 was not active alone, or with complement, but it mediated antibody dependent cell cytotoxicity (ADCC) against several different types tumor cells *in vitro*. Surprisingly, ESK1 antibody substantially reduced established disseminated, human leukemias in NSG mouse models, at doses as low as 50 to 100 ug. Therapeutic effects were more pronounced and more durable when combined with human CD34-, CD3-, human NK

and monocyte effectors in the NSG mice. We concluded that ESK1 is a potential therapeutic agent for a wide range of human leukemias and cancers over-expressing the WT1 oncoprotein.

In order to make the ESK1 more potent, we engineered a second-generation mAb, ESKM, which displayed enhanced antibody dependent cell-mediated cytotoxicity (ADCC) function due to altered fucosylation on the Fc. In ADCC assays *in vitro* ESKM was more potent than native ESK1 against a variety of cancers including mesothelioma and leukemia. While ESK antibodies mediated ADCC against several hematopoietic cancer cell lines and fresh cancer cells and solid tumor cells at concentrations below 1μg/ml, but ESKM was about 5-10 fold more potent *in vitro* against these multiple cancer cell lines. ESKM also was more potent *in vivo* against JMN mesothelioma. ESK was effective against SET2 AML and fresh ALL xenografts. HLA-A**02:01+* transgenic mice were used to assess ESKM toxicity. No changes in cell numbers or cell types in the blood spleen or BM were observed, including murine stem cells. ESKM had a shorter half-life (4.9 *vs* 6.5 days). The biodistribution pattern in C57BL6/J mice was the same as the native IgG1. There was no difference in half-life or biodistribution in HLA-A**02:01+* transgenic mice compared to the parent strain, at therapeutic doses of ESKM. Therapeutic doses of ESKM in these mice caused no pathologic tissue damage. These data support the concept that an Fc-enhanced mAb can improve efficacy against a low-density, tumor-specific, peptide/MHC target. We plan to develop such an mAb against this important intracellular oncogenic protein.

Additional experiment were done to try other formats of the ESK mAb that might be even more effective. One new effective immunotherapy for cancer that directs T cell cytotoxicity against cells expressing a cell surface protein is recognized by an antibody scFv is Bi-specific T cell engager antibody (BiTE) therapy. Therefore, we generated the first BiTE

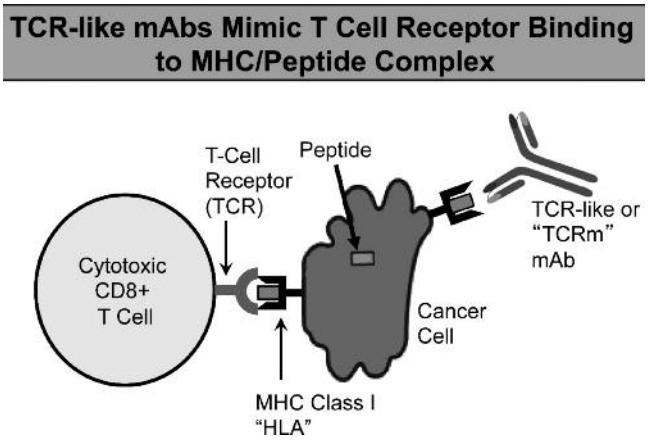

Figure 1 Schematic of TCrm mAb function

construct derived from our TCR-mimic ESK mAb. ESK-BiTE was able to selectively activate and induce proliferation of cytolytic human T cells. ESK-BiTE was also able to kill multiple leukemias and cancers *in vitro* and in mice. These activities occurred despite the ultra-low density at the cell surface of the peptide/HLA-A2 complex.

In an effort to add multi-functionality and multi-valency to our antbody platform we also explored use of nanomaterial carriers. We developed a nanoscale isotope generator that elaborates 4 alpha particle emitting elemnts at the tumor cell site[4,5]. This targeted nanogenerator is the most potent cancer cytotoxic agent known, requiring only 1 delivered alpha to kill a cell.

Single-walled carbon nanotubes (SWNTs) provided an even more versatile platform. SWNT can deliver imaging agents or drugs to tumors and offer several advantages over approaches based other nanomaterials or antibodies. The nanotubes can carry 100 times more cargo than an mAb, but are still rapidly eliminated from circulation by renal filtration, like a small molecule, due to their narrow dimension of 1 -2 nanometers and their

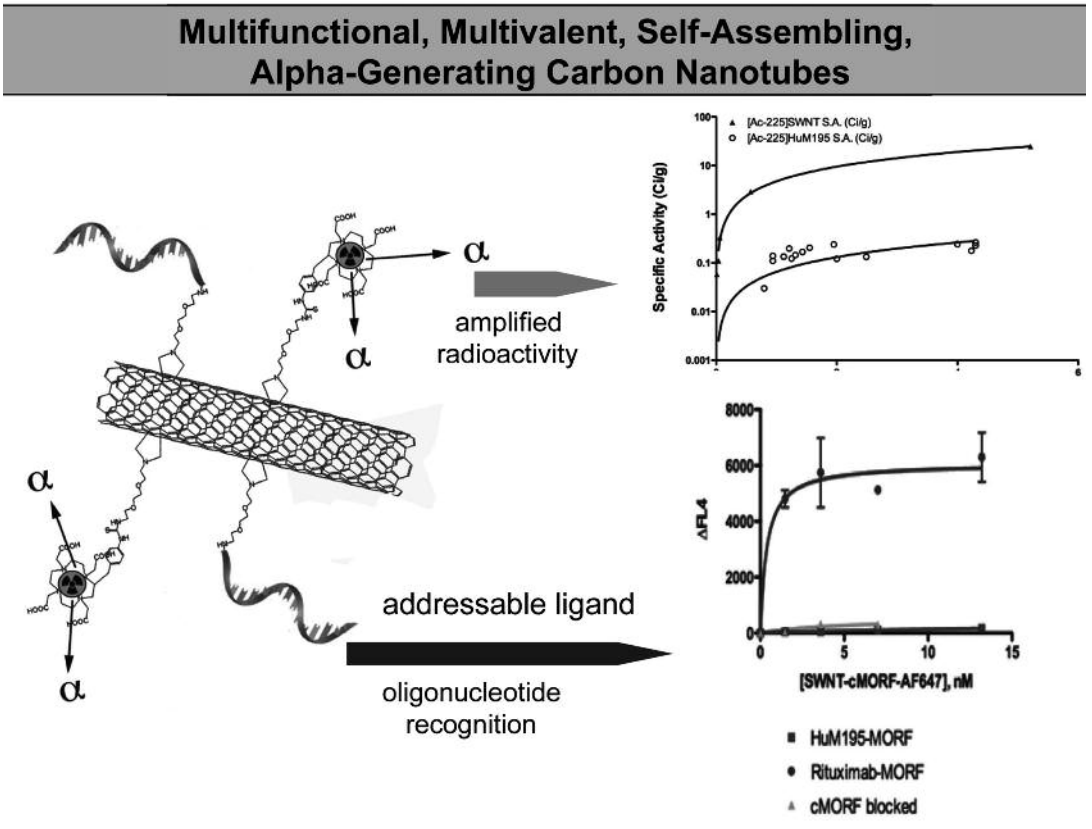

Figure 2 SWNT can be appended with multiple ligands (left). One hundred times more Ac-225 can be attached to a SWNT than an mAb (upper right). Use of morpholinos allows rapid, high affinity addressable labels to assemble the parts (lower right).

high aspect ratio of 300:1. Nanotubes can carry fluorophores or radioisotopes, and were shown to selectively bind to cancer cells *in vitro* and in tumour-bearing xenografted mice. SWNT conjugates can be labelled with both high specific activity alpha-particle and gamma-ray emitting isotopes for imaging. Using nanotubes modified with morpholino oligonucleotide sequences to bind to cancer cells that have been pre-targeted with antibodies modified with oligonucleotide strands complementary to those on the nanotubes, we showed that SWNTs can target tumors in a two-component self-assembling approach[6-8]. (Figure 2)

The binding of the SWNT was also found to lead to internalization of the antibody/nanotube complexes. Because the SWNT conjugates labelled with alpha-particle generating 225 Ac were found to clear rapidly, unlike the slower clearing mAB, radioisotope toxicity was mitigated. The two step self assembly process was shown to be therapeutically effective *in vivo*. Thus using SWNT-oligonucleotide conjugates as self-assembling, tumor-targeted devices may be feasible.

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2012-	Tri-institutional Program in Chemical Biology; Faculty member, SKI
2013-2013	Director (interim) Sloan-Kettering Institute
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WT1 (WILM'S TUMOR GENE) PEPTIDE CANCER VACCINE

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Wilms' tumor gene 1 (WT1) is isolated as a gene responsible for childhood renal tumor, Wilms' tumor, encodes a transcription factor that regulate many genes, and plays an important role in cell proliferation and differentiation, embryogenesis, leukemogenesis and tumorigenesis.

I discovered that WT1 was a good molecular marker for detection of minimal residual leukemic cells in leukemia patients[1]. Quantitation of WT1 mRNA (WT1 mRNA assay) in peripheral blood mononuclear cells made it possible to detect one leukemic cell in 100,000 normal mononuclear cells. WT1 mRNA assay becomes an essential clinical test for treatment of leukemia and myelodysplastic syndrome (MDS) and is commercially available in Japan and EU, and soon in USA. WT1 mRNA assay is covered by national health insurance in 2007 for AML and in 2011 for MDS in Japan.

I also discovered that WT1 protein was expressed in not only leukemia but also almost all types of solid tumors and thus was a pan-tumor associated antigen. I identified a WT1235 (235-243 aa) and WT1126 (126-134 aa) peptides as an HLA-A*2402- or HLA-A*0201- restricted WT1 peptide, respectively. I started for the first time (First – in – Man) a phase 1 clinical study of WT1 vaccination for patients with leukemia, MDS, lung cancer, or breast cancer in 2001[2]. Patients were intradermally injected with an HLA-A*2402-restricted, natural (CMTWNQMNL) or modified (CYTWNQMNL) 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant at 0.3, 1.0, or 3.0 mg per body at 2-week intervals, with toxicity and clinical and immunological responses. Toxicity consisted only of local erythema at the WT1 vaccine injection sites. Three of the four AML patients were successively being injected WT1 vaccine and are in complete remission over 11 years. A

phase II clinical study, in which WT1 vaccination was intensified and repeated every week at a dose of 3.0 mg / body of modified WT1 peptide for three months, were started from 2004, and safety of this study was confirmed. Clinical effect of WT1 vaccination is satisfactory for glioblastoma multiforme with relapse and disease control rate is 47.7%[3]. Adverse effect is only erythema at WT1 vaccine injection sites. Thirty-two patients with advanced pancreatic cancer were biweekly WT1-vaccinated and received gemcitabine(1000 mg/m2) on days 1, 8, and 15 of a 28-day cycle. This combination therapy was well tolerated. The frequencies of grades 3-4 adverse events for this combination therapy were similar to those for gemcitabine alone. Objective response rate and disease control rate was 20.0% (6/30 evaluable patients) and 75.0%, respectively. Clinical response was 7 PR (21.9%), 17 SD (53.1%), 7 PD (21.9%) and 1 unevaluable. Median survival rate was 8.1 months and 1-year survival rate was 29%. We have been performing randomized phase II clinical study of WT1 vaccine +gemcitabine vs. gemcitabine alone[4].

Until now, over 750 patients with AML, CML, MDS, myeloma, glioblastoma multiform, pancreatic, lung, breast, thyroid, colon, gynecological, renal, or other cancers were WT1 vaccinated. However, there is no WT1 vaccination-related death, indicating that WT1 vaccination is very safe.

The National Cancer Institute of USA evaluated 75 popular tumor associated antigens for the clinical utility and ranked WT1 antigen as a top among 75 tumor associated antigens[5].

In cure-oriented cancer therapy, complete eradication of quiescent cancer stem cells is essential. Only immunity against cancer is considered to be able to eradicate non-dividing quiescent cancer stem cells. Therefore, we proposed a novel strategy for cancer treatment as follows. At present time, WT1 peptide vaccine is usually used as the last line treatment under the impaired immune conditions of cancer-bearing patients and thus immune response induced is limited to weak. In a novel strategy, WT1 peptide vaccination will be started as soon as possible when patients were diagnosed as having cancer and on the base line of WT1 immunity induced, surgery, chemotherapy and /or radiotherapy should be performed. In such situation, for example, in the combination of WT1 vaccination and chemotherapy, some chemotherapies sometimes dominantly suppress immune suppressive cells, resulting in relative increase in WT1 immunity against cancers.

Nowadays, two WT1 killer peptide vaccines (one for HLA-A*24:02+ patients and another for HLA-A*02:01+ patients) and one WT1 helper peptide vaccine(HLA class II-restricted, but universal peptide) are under phases I and II clinical studies, respectively, by two pharmaceutical companies.

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1994-1995	Lecturer, Department of Medicine III, Osaka University Medical School
1995	Professor, Department of Functional Diagnostic Science, Osaka University Medical School
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Present	Professor and Chairman, Departments of Functional Diagnostic Science, of Cancer Vaccine, of Cancer Stem Cell Biology and of Cancer Immunology, Osaka University

Specialty and Present Interest:
Tumor Immunology, Hematology

SYNERGY OF THERAPEUTIC VACCINATION AGAINST HPV16 ONCOGENIC PROTEINS AND STANDARD CHEMOTHERAPEUTICS

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We previously developed a vaccine, comprising human papillomavirus (HPV) type 16 E6 and E7 overlapping synthetic long peptides (HPV16-SLP), that was clinically active in patients with HPV16$^+$ high-grade vulvar intraepithelial neoplasia[1)]. Complete lesion regression correlated with a strong vaccine-prompted HPV16-specific effector T-cell response[1,2)]. However, in patients with HPV16-induced metastatic cervical cancer vaccine-induced T-cell responses were weaker and did not result in improved clinical outcome[3,4)], although some patients benefited clinically from subsequent treatment with chemotherapeutic agents[3)], suggesting that the combination of both modalities can make a difference. In a preclinical mouse model of HPV16-induced cervical cancer we studied whether SLP vaccination could be combined with chemotherapy to eradicate tumors. First we tested the effect of SLP vaccination in combination with individual chemotherapeutic agents. In most mice that received either peptide vaccination or chemotherapy treatment, only a temporary regression in tumor size was observed. Importantly, combined chemo-

immunotherapy induced complete tumor eradication in nearly all mice[5]. The chemotherapeutic agents cisplatin and carboplatin displayed the strongest synergy with SLP vaccination. This synergy was not due to increased sensitivity of cisplatin treated tumor cells to CTL-mediated killing, or to a stronger vaccine-induced circulating tumor-specific T cell response. Analysis of the intra-tumoral immune response revealed that combined treatment with cisplatin and SLP vaccination resulted in a strong increase in the density of intra-tumoral, Tumor Necrosis Factor alpha (TNFα) and Interferon gamma (IFN-γ) producing $CD8^+$ CTLs. Tumor cells incubated with TNFα and IFN-γ, together with cisplatin strongly enhance their chemokine expression, compatible with the abundant leukocyte infiltration in the tumor upon combined chemo-immunotherapy. Accordingly, when combined with systemic cisplatin treatment, SLP vaccine-induced CTLs appeared to migrate earlier into the tumor beds. Moreover, analysis of the tumor cells *in vivo* showed that combined treatment not only caused a decrease in the proliferative capacity of tumor cells, but also a TNFα- induced enhancement of cisplatin- mediated tumor cell death. Cell death was accompanied by an increased expression of pro-apoptotic molecules. Importantly, when SLP vaccination and cisplatin were combined, the synergistic effect of this combination on tumor eradication *in vivo* was abolished by treatment of the mice with i.p. injections of monoclonal antibody against TNFα[5]. We then tested the effect of SLP vaccination together with the combination carboplatin and paclitaxel, which is a standard of care clinical chemotherapy doublet. It was observed that abnormally high mononuclear myeloid cell populations found in the blood and in tumors of mice were normalized to much lower levels by this chemotherapy doublet, again permitting a marked increase in the response to SLP vaccination and showing synergy in tumor eradication. A new clinical pilot study on the constitution of immune cells in the blood of late stage cervical cancer patients also revealed increased numbers of mononuclear myeloid cells, associated with low T-cell reactivity against common microbial recall antigens and a lower stimulatory capacity of antigen presenting cells, indicating an immunosuppressed status. When these patients were subsequently treated with the standard carboplatin-paclitaxel chemotherapy a normalized systemic immune profile similar to that found in healthy subjects was observed at a specific time point during this chemotherapy. Together these data indicate that standard chemotherapy has a variety of immunostimulatory effects, including better attraction of T cells into tumors and greater sensitivity of tumors to apoptosis by TNFα produced by T cells as well as better expansion of T cells through depletion of myeloid derived suppressor cells without suppression of T cell function or numbers. These combined effects lie at the basis of synergy between SLP vaccination and chemotherapy in mouse tumor eradication. Therefore a pilot clinical trial was started in which 12 late stage cervical cancer patients were treated with standard chemotherapy in combination with a

single dose of HPV16-SLP vaccination. Comprehensive immune monitoring confirmed the beneficial effect of myeloid cell depletion associated with a robust induction of HPV16-specific T-cell responses after the single dose of HPV16-SLP vaccination, that were sustained throughout several cycles of chemotherapy. Currently, we have started a multicenter trial in which HPV16-SLP vaccination is combined with carboplatin-paclitaxel and type I interferon to assess clinical and immunological outcome in a group of $HPV16^+$ metastatic cervical cancer patients. The treatment scheme of this trial is shown in Table 1. Each patient cohort consists of 6 patients with recurrent or metastatic cervical cancer. 4 dose levels of HPV16-SLP vaccine (termed ISA101) will be given and at each dose level patients will receive pegylated interferon alpha 2b (Pegintron) or not. Interferon alpha 2b is expected to promote the CD8 component in the response and to promote dendritic cell activation for increase in both CD4 and CD8 responses.

Table 1 As of this writing (November 2014), 18 patients have been recruited into the study. The results will be reported in due course.

Therapy / Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Carboplatin + paclitaxel	X Cycle 1			X Cycle 2			X Cycle 3			X Cycle 4			X Cycle 5			X Cycle 6		
ISA101 ± pegylated IFNα						X Vac. 1			X Vac. 2			X Vac.3						

Cohort	# Patients	Carboplatin / Paclitaxel	ISA101	Pegylated IFNα
01	6	AUC 6 / 175 mg/m²	20 µg/peptide	-
02	6	AUC 6 / 175 mg/m²	20 µg/peptide	1 µg/kg
03	6	AUC 6 / 175 mg/m²	40 µg/peptide	-
04	6	AUC 6 / 175 mg/m²	40 µg/peptide	1 µg/kg
05	6	AUC 6 / 175 mg/m²	100 µg/peptide	-
06	6	AUC 6 / 175 mg/m²	100 µg/peptide	1 µg/kg
07	6	AUC 6 / 175 mg/m²	300 µg/peptide	-
08	6	AUC 6 / 175 mg/m²	300 µg/peptide	1 µg/kg

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CANCER IMMUNOTHERAPY: THE NARROW ROAD BETWEEN INEFFICACY AND TOXICITY

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It is now established that most melanoma patients produce a spontaneous T cell response against their tumor. In many patients this attempt at eliminating the tumor evidently fails and a large number of anti-tumor T cells remain in an inactive state in the circulation and within tumor deposits. Inside the tumors, this anergic state appears to be reinforced by an immunosuppressive environment. For the vaccinated patients who show tumor regression, we propose the following sequence of events: a very small number of active anti-vaccine T cells penetrates the tumor and attacks some tumor cells. As a result, these CTL are restimulated and produce cytokines which focally reverse the local immunosuppression, creating instead an immunostimulatory environment. This reawakens many of the inactive anti-tumor T cells that are already present in significant numbers *in situ*. Naïve anti-tumor T cells may also be activated. The mobilization of all these T cells, most of which are directed against tumor antigens other than the vaccine antigen, provides the large numbers of effector cells required to reject the tumor.

Why is it that following therapeutic vaccination with tumor-specific antigens tumors regress in a small minority of patients whereas no sign of regression is observed in most patients? A first possibility is that this may be due to differences in the quantity or quality of T cells responding to the vaccine. However, the lack of improvement of clinical outcome following highly immunogenic vaccination suggests that the strength of the response to the vaccine is not the limiting factor. A second possible difference between regressing and non-regressing patients could be the degree of expression of the relevant antigens on the surface of tumor cells. It is well established that tumors can lose the expression of some or all of their HLA molecules. However, we derived melanoma cell lines from a large number

of non-responder patients, and almost all of them proved to be sensitive to anti-vaccine CTL *in vitro*. We therefore suspect that loss of antigen expression accounts for only a small fraction of the treatment failures. A third possible explanation for the individual differences in clinical response is that patients vary in the degree of immunosuppression present in their tumor. A highly immunosuppressive tumor environment could render the anti-vaccine T cells inactive soon after penetrating the tumor, preventing them from initiating the cascade of events that leads to tumor rejection. The responder patients would be those who have a lower degree of immunosuppression in their tumor.

The anergic state of tumor-infiltrating lymphocytes and the immunosuppressive tumor environment are often considered to result from an active immunosuppressive mechanism of tumor cells acquired in the course of the selection for resistance to the immune response. Agents such as IDO, TDO and TGF-β are produced in many tumors and unquestionably contribute to inducing anergy of infiltrating lymphocytes. But it is also possible that when the immune system makes a rather weak attempt to eliminate a tissue, as expected in the absence of pathogen-associated molecular patterns binding to Toll-like, Nod and RIG receptors, after a length of time it enters an inactive state. Examples of such "immune fatigue" are observed in human transplantation: a small number of recipients can be withdrawn from treatment with immunosuppressive agents without deleterious effects on the grafted organ. This process, whereby an incomplete response evolves into an anergic state could be common to all progressive metastatic tumors, whereas the presence and the nature of immunosuppressive factors secreted by tumor cells are likely to vary from one tumor to another. The common anergic state could involve a degree of immunosuppression within the tumor resulting in potential paralysis of incoming T lymphocytes.

If the degree of intratumor immunosuppression is the limiting factor for the success of vaccination against tumors, the use of cytokines to alleviate this immunosuppression could improve the outcome. We feel that local cytokine treatment of metastases should be tested in the clinic as an adjunct to vaccination. This local treatment could enable the anti-vaccine T cells to initiate the rejection process. Moreover, the amount of agents used for local treatment would be orders of magnitude below those used for systemic treatments, thereby reducing the occurrence of adverse side-effects. We realize that this approach will lead to therapeutic progress only if the immune rejection response of the treated metastasis leads to the production of anti-tumor T cells of such quantity and quality that they successfully attack untreated metastases disseminated elsewhere in the body.

We carried out preclinical studies using the mouse HY system, which involves T cell responses to antigens expressed in male and not in female tissues. This model is attractive as it has several features shared with the immune response to human tumors. Whilst in

inbred mouse strain C57BL/6 ($H2^b$) females reject syngeneic male skin grafts, in CBA ($H2^k$) they do not, even though we have observed that a CTL response directed against HY antigens does occur following grafting. Thus the process observed in CBA is similar to that observed in progressing human tumors: a failed attempt at rejection followed by the persistence of ineffective memory T cells. We observed that local immunostimulatory treatments involving certain combinations of cytokines and TLR ligands can reactivate stalled T cell responses against male mouse skin grafts resulting in graft rejection. Local injections of a mixture of low-dose IL-12 and interferon alpha proved effective but required prolonged treatment. Similar results were obtained with a combination of Gardiquimod , a TLR7 ligand , and IL-2.

The purpose of this report is to stimulate clinical cancer immunotherapy research combining vaccination with local and systemic immunostimulatory agents. We are convinced that local immunostimulation should be supplemented with vaccination against specific tumor antigens. The influx into the tumor of anti-tumor T cells, which have been freshly produced by the vaccination and therefore have not yet been rendered anergic, may prove to be a key condition for success. Considering the absence of toxicity observed with anti-tumor vaccination and the extremely low dose of immunostimulatory agents applied locally, we expect this combination treatment to produce very little or no toxicity. The combinations of immunostimulatory agents described here could be used in a first step. Additional agents could be put into play later according to the observations that will be made. Agents that counteract immunosuppressive factors produced or elicited by cancer cells may be particularly useful. A low systemic dosage of anti-PD1, anti-PDL1 or anti-CTLA-4 might provide added efficacy without provoking autoimmune side effects. Perhaps our cancer immunotherapy strategies should emulate the immune system by administering a multiplicity of agents that complement each other.

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Specialty and Present Interest:
Immunology, Immunotherapy, Melanoma, Cancer Vaccines

OPTIMIZING THERAPEUTIC CANCER VACCINES

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Therapeutic cancer vaccination aims at boosting naturally acquired as well as priming *de novo* tumor antigen specific T cell responses that may control and/or eliminate tumors. The molecular characterization of T cell-defined tumor antigens in the early 90s opened the way to design vaccines based on targeting one or more well defined tumor antigens.

Extensive preclinical and clinical testing of a large variety of vaccine formulations addressed to many types of cancer has provided a wealth of information. Modern vaccination consistently elicits tumor specific T cell responses in the majority of treated cancer patients. Clinical benefit is achieved in only a proportion of those patients and the best estimates indicate that one in four patients would experience disease stabilization, mixed tumor responses or even objective tumor regression. Two or three cancer vaccines have been approved for clinical use in three countries and there are at least 15 vaccines in pivotal clinical trials. However, some of them have recently failed their end points despite the promising signals in the phase II clinical trials. There is therefore a need to improve vaccines and optimize vaccination strategies so as to reach consistent clinical efficacy.

In our studies, we have identified several parameters for vaccine optimization. Peptide-based vaccines can be enhanced via the substitution of selected amino acid residues that increase immunogenicity yet preserve antigenicity and tumor reactivity[1,2)]. Long synthetic peptides effectively target professional antigen presenting cells and may offer both class I and class II restricted T cell epitopes. Subunit vaccines are strictly dependent on the co-administration of potent immunological adjuvants. TLR agonists are effective adjuvants that signal dendritic cell activation and maturation. Both poly(I:C), a double stranded RNA analog interacting with TLR-3, and CpG-oligodeoxynucleotides, ligands for TLR-9, are

potent in both pre-clinical models and in cancer patients[3,4]. Invariant NKT cells may also provide strong immunological adjuvant activity[5]. Further, vaccine optimization efforts are currently focused on identifying approaches to improve and sustain T cell function in the strongly suppressive tumor microenvironment[6], as well as in understanding the molecular mechanisms underlying the induction of long lived memory T cells that may provide robust immunity over prolonged periods of time[7].

Different adjuvants differ in their ability to induce a high CD8 T cell effector to regulatory T cell ratio in response to peptide immunization:

We used a double adoptive transfer of TCR transgenic T cells specific for class I and class II restricted peptide antigens in which the latter was also knocked in for a eGFP-Foxp3 reporter gene. Tracking in this way tumor-specific CD8, CD4 and Treg responses to pepti in varios adjuvant formulations, we could show that vaccines containing TLR-9 CpG-ODN or TLR-3 ligand poly(I:C) preferentially induce strong proliferation of antigen-specific effector T cells, while minimizing expansion of antigen-specific Tregs. High Teff:Treg rations were linked to strong Th1 cytokine production in the lymph nodes early after immunization and resulted in polyfunctional CTLs with enhanced tumor infiltration and protective function (Figure 1)[3].

Targeting the cross-presenting dendritic cell subset improves polypeptide vaccination:

Therapeutic cancer vaccines based on synthetic peptides have provided encouraging clinical signals in both intravaginal neoplasia (HPV long synthetic peptide cocktail)[8] and metastatic renal cell carcinoma (synthetic naturally presented MHC-I restricted peptides)[9]. However, the same cocktail of HPV antigenic long synthetic peptides vaccine failed to modify tumor progression when used to vaccinate advanced cervical carcinoma patients. Moreover, two phase III randomized placebo-controlled clinical trials of vaccination with a

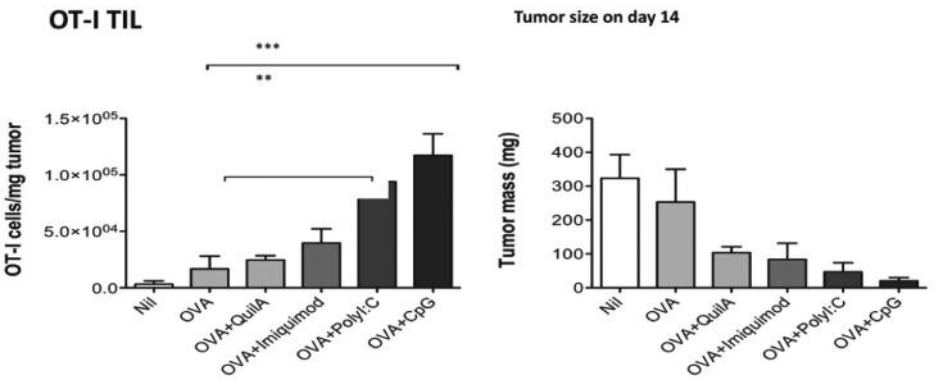

Figure 1 OT-I Teff accumulation in TIL depends on the adjuvant used and correlates with tumor protection.

MAGE-A3 recombinant fusion protein failed their primary efficacy endpoints in locally advanced local carcinoma and in metastatic melanoma. In these cases, no detectable tumor protection was accompanied by modest induction of specific CD8 T cell responses. A possible explanation for these outcomes is the limited cross-priming of T cell immunity by soluble polypeptide antigen. It would be desirable to target these antigens to the unique dendritic cell subset specialized in cross-presentation. This cell type is present in spleen and in dermal dendritic cells at low frequencies, is dependent on the master transcription factor Batf3 and is characterized by the expression of CD8αα homodimers, CD141, CLEC9A and high levels of TLR3. The cross-presenting DCs are the majority of cells outlined by this phenotype and can be identified by their expression of XCR1 chemokine receptor (Figure 2). Its ligand, XCL1, is unique among the chemokine families and is abundantly produced by activated CD8 T cells. It has been suggested that targeting antigens through this ligand-receptor pair may increase the cross-priming of specific CD8 T cells to soluble protein immunogens[10]. We have used either the free XCL1 polypeptide or Fc-XCL1 recombinant fusion proteins to assess the impact on the relative immunogenicity of soluble recombinant protein or long synthetic peptide and TLR agonist as compared to immunization with TLR agonist alone. The results indicate that immunization with XCL1 leads to increased frequencies of specific CD8 T cells, enhanced recall responses and

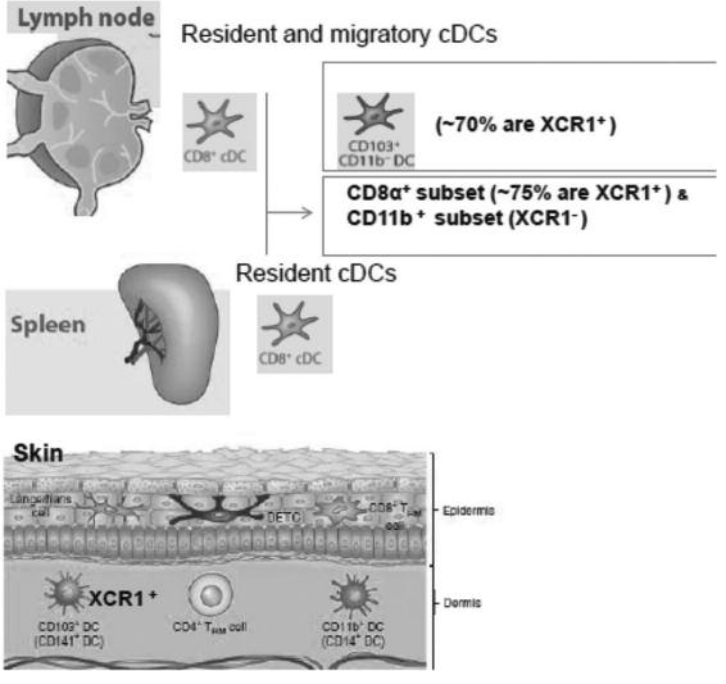

Figure 2 Cross priming dendritic cells.
The human orthologues are BDCA3⁺ (CD141, thrombomodulin), CLEC9A⁺, XCR1⁺ and TLR3⁺. Adapted from Merad et al, 2013 & Heath et al, 2013.

increased functionality. Importantly, preliminary results also suggest that therapeutic vaccination including the XCL1 chemokine may lead to enhanced ability of the responding CD8 T cells to control tumor growth in a model of OVA-expressing lymphoma (Figure 3).

Understanding molecular switches steering T cell differentiation:

It has been shown that feeding rapamycin to mice acutely infected with the lymphochoriomeningitis virus (LCMV) may increase the quantity and/or quality of LCMV antigen-specific CD8 T cells[11]. This effect has been confirmed in additional models of infection and of vaccination with recombinant viral vectors. Increasing the memory T cell responses induced by vaccination with tumor antigens may contribute to long lived tumor protection. Since rapamycin selectively inhibits mTORC1 in several cell types but also mTORC2 in others, we have set out to dissect the role of mTORC1 and mTORC2 in regulating T cell differentiation using conditional deletion of Raptor and Rictor in T cells. A mouse line in which Raptor, a regulatory component of the mTORC1 complex, was deleted in T cells had a profoundly reduced effector CD8 T cell response in response to infection with LCMV. The few effector phenotype cells generated displayed severely reduced cytokine secretion in response to antigenic peptide challenge and had reduced content of perforin and granzymes. Moreover, the memory response was also nearly absent in comparison to mTORC1 competent littermates (Figure 4). Thus, in contrast to the effect of mTORC1 inhibition mediated by rapamycin, genetically mediated ablation of the mTORC1 signaling led to near complete inhibition of the memory response as well as of the acute effector CD8 T cell response. Preliminary results with the mouse line with a conditional deletion of Rictor, a key regulatory component of the mTORC2 complex, suggest a twofold reduction in the effector CD8 T cell response accompanied by an accelerated induction of higher frequencies of memory CD8 T cells. The latter appeared functionally competent and able to mediate enhanced recall responses to antigen as compared to the Rictor wild type littermates. Experiments in progress explore the mechanisms involved as well as the relationship between the effect of rapamycin on CD8 T cell differentiation and that of genetic modulation of the two branches of mTORC signaling. Moreover, we are also performing metabolic profiling of differentiating CD8 T cells to understand the extent to which mTOR signaling mediated metabolic changes in differentiating T cells determine their ultimate functional fates. We expect that insights from these studies would inform the design of cancer vaccines able to induce optimized CD8 T cell responses capable of mediating potent, long lived protective anti-tumor immunity.

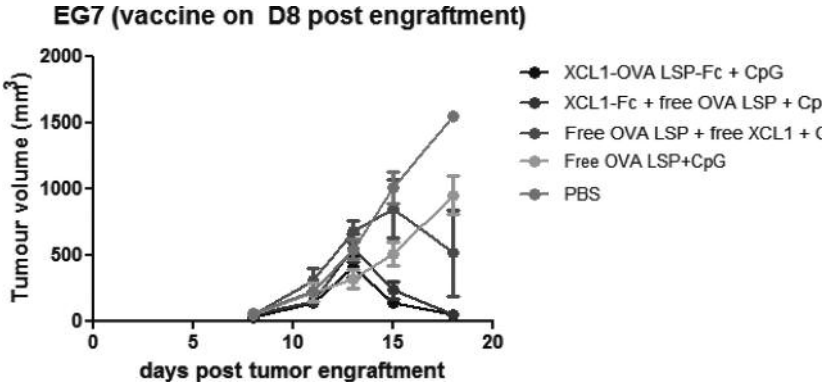

Figure 3 XCL1 enhances CD8 T cell induction by vaccination with long synthetic peptide.

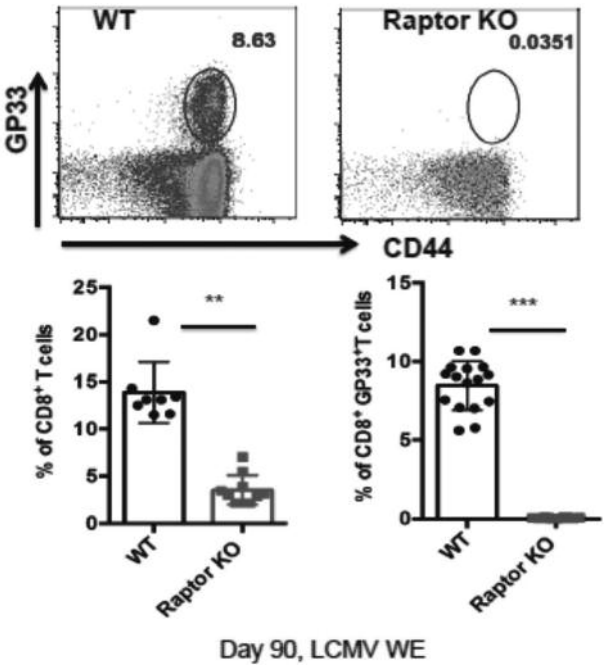

Figure 4 Loss of specific CD8 T cell memory formation in the absence of Raptor.

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Specialty and Present Interest:
Immunology, Tumor Immunology and Immunotherapy of Cancer

CANCER IMMUNOTHERAPY USING NOVEL TUMOR-ASSOCIATED ANTIGENIC PEPTIDES AND HUMAN iPS CELL-DERIVED DENDRITIC CELLS

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Y. Nakamura and his colleagues analyzed the gene expression profiles of various cancerous and normal tissues using a genome-wide cDNA microarray covering more than 27,000 genes, and subsequently many novel oncofetal or cancer testis tumor-associated antigens (TAAs) were identified[1-6]. These TAAs are overexpressed in human malignant tumors including head and neck squamous cell cancer (HNSCC), esophageal, lung, pancreatic, and urinary bladder cancers etc., but not in many adults normal tissues except for testis, placenta or fetal organs. Furthermore, these TAAs are involved in promotion of malignant cell proliferation and the high expressions of TAAs were correlated with poor prognosis of the patients.

Subsequently we have identified many these TAAs-derived short peptides (SPs) recognized by cytotoxic T lymphocytes (CTLs) in the context of Japanese common HLA-A2 or -A24 by using HLA transgenic mice *in vivo* and human PBMCs *in vitro* (Figure 1)[1~6]. Moreover we succeeded in propagation *in vivo* of TAAs-derived SPs-specific CTLs in the majority of patients with HNSCC by immunization with these SPs without any serious adverse effects in a phase II clinical trial with 37 HNSCC patients. Positive clinical responses including complete response were observed in a fraction of patients and the strong correlation between magnitude of CTL responses induced in the vaccinated patients and prolonged overall survival periods was also observed[7]. Now the phase II clinical trial, aiming at prevention of recurrence and metastasis of HNSCC has been started in patients who have received curative operations.

It is well known that $CD4^+$ T cells have important role in the immune system and promote effective T-cell-mediated anti-tumor immunity. $CD4^+$ helper T (Th) cells are

essential for sensitization of tumor-specific $CD8^+$ T-cells to induce the expansion and differentiation of TAA-derived SPs-specific CTLs, and generation and maintenance of long-lasting CTL responses[8]. Th cells also have a crucial role to pave the way for entry of CTLs at the tumor microenvironments. Especially T-helper type 1 (Th1) cells have a crucial role in elimination of tumor and IFN-γ produced by Th1 cells exhibits anti-angiogenic effects. Therefore, it is important to identify TAA-derived peptides that can activate not only CTLs but also tumor-specific Th1 cells for induction of effective tumor immunity in tumor-bearing hosts. Professional antigen presenting cells including dendritic cells (DCs) has a capacity to process extracellular antigens and present antigens-derived SPs in the context of HLA class I molecules via so called cross-presentation pathway leading to the activation of a TAA-derived SPs-specific CTL responses. Recent clinical study have shown that targeting Th cells and CTLs using both HLA class I- and II-restricted epitopes enhanced vaccine-specific immune responses and improved clinical responses[9]. As a result, a single polypeptide encompassing epitopes for both Th1 cells and CTLs may be an ideal peptide

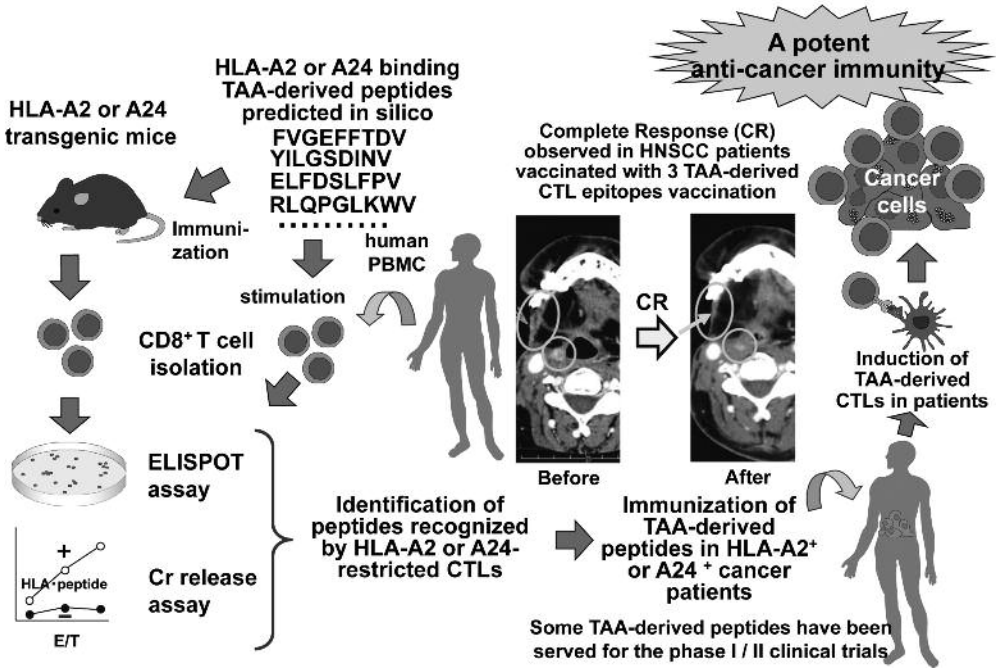

Figure 1 Identification of tumor-associated antigens (TAAs)-derived short peptides (SPs) carrying cytotoxic T lymphocyte (CTL) epitopes using HLA-class I transgenic mice and their application to the clinical trials of cancer immunotherapy.

We have identified many TAAs-derived SPs recognized by cytotoxic T lymphocytes (CTLs) in the context of Japanese common HLA-A2 or -A24 by using HLA transgenic mice *in vivo* and human PBMCs *in vitro*. We have succeeded in induction of SPs-specific CTL in many head and neck squamous cell cancer patients and a prolongation of survival period in a small fraction of patients by vaccination with three CT antigens-derived SPs.

vaccine for cancer immunotherapy.

Based on these information, we attempted to identify TAA-derived long peptides (LPs) that can induce both TAA-specific Th1 cells and CTLs in order to further improve TAA peptides-based cancer immunotherapy. We focused on three cancer testis antigens, Cell division cycle associated 1 (CDCA1), Kinesin family member 20A (KIF20A) and Lymphocyte antigen 6 complex, locus K (LY6K). We have identified highly immunogenic TAAs-derived LPs encompassing both Th1 cell- and CTL-epitopes. The 24-26 mer LPs encompassed naturally processed Th cell epitopes which are presented by various frequent HLA-class II molecules (Figure 2). Based on the findings, CDCA1-, KIF20A- and LY6K-LPs are useful in at least 90% of the Japanese population[10-12]. These Th1-cell epitopes can induce CTLs as well as Th1 cells in both human *in vitro* and HLA class I transgenic mice *in vivo* by possible crosspresentation. Moreover we observed an augmentation of TAA-specific Th1 cell responses in cancer patients immunized with TAA-derived CTL epitope

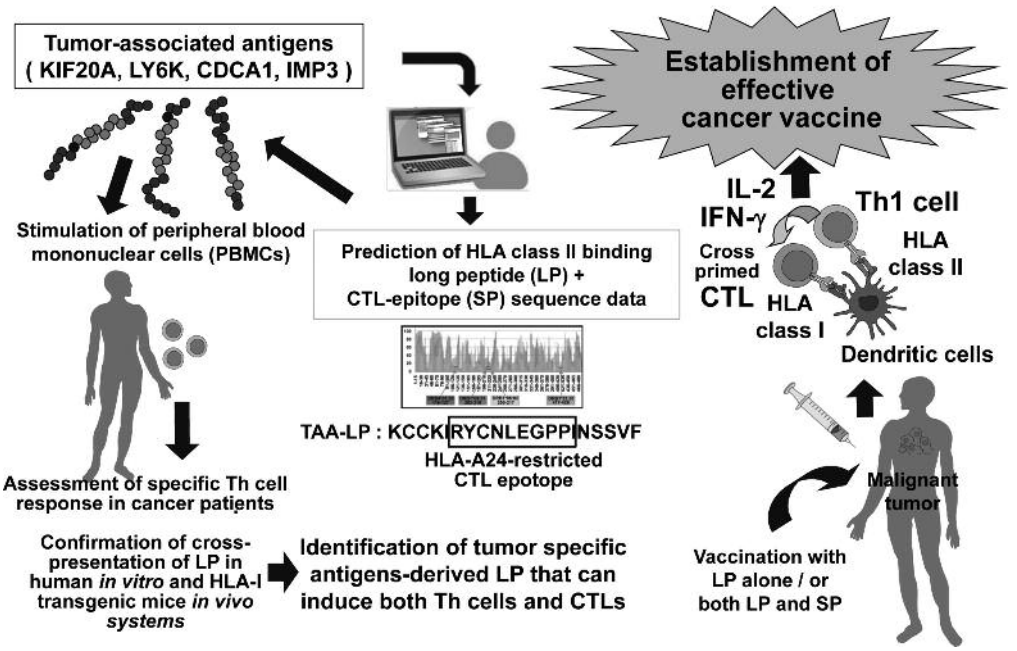

Figure 2 Development of effective cancer immunotherapy using TAA-derived long peptides carrying both HLA-II restricted Th1 cell and CTL epitopes.

We attempted to identify TAA-derived long peptides (LPs) that can induce both TAA-derived LPs-specific Th1 cells and TAA-derived SPs-specific CTLs in order to further improve TAA peptides-based cancer immunotherapy. We have succeeded in identification of highly immunogenic TAAs-derived LPs encompassing both Th1 cell- and CTL-epitopes.

SPs vaccines.

We observed the synergistic effect of these 3 TAA-derived LPs on induction of antigen-specific and CD107a-positive CTLs. These findings indicate that the LPs may be able to augment the induction of antigen-specific CTLs in combination with immunotherapy using CTL-epitope SPs and improve clinical outcomes in cancer expressing TAAs. The checkpoint blockade by specific antibodies to cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death -1 (PD1), or it's ligand PD-L1 etc. in combination with therapeutic vaccines has been illustrated[13]. Thus, checkpoint blockade may be a good candidate for combination therapy with TAAs-derived LPs vaccine.

We previously succeeded in generation of immune competent DCs from both mouse and human ES (ES-DC) and iPS cells (iPS-DC)[14–18]. In mouse model, these ES-DC and iPS-DC stimulated both TAA-specific CTLs and Th cells to inhibit tumor growth *in vivo*[14, 16]. Human iPS-DC also activated HLA-I-restricted CTLs reactive to TAA-derived SPs to kill tumor cells *in vitro*. Moreover, we succeeded in generation of TAP-deficient human iPS-DCs expressing an allogeneic HLA-A2 gene, and these iPS-DCs could induce HLA-A2-

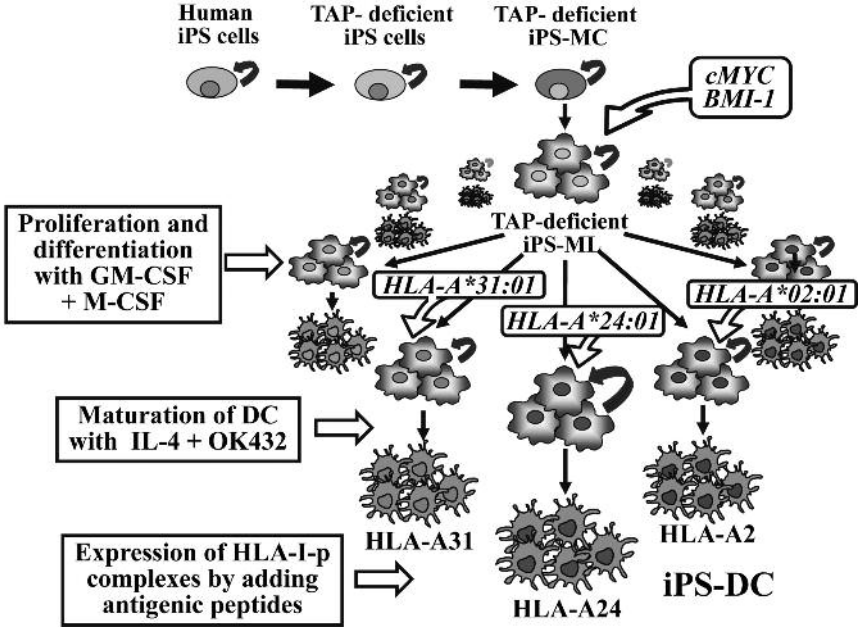

Figure 3 Targeted disruption of TAP2 gene in human iPS cells to generate universally applicable dendritic cell source.

We have succeeded in generation of TAP-deficient human iPS-DCs expressing an allogeneic HLA-A2 gene, and these iPS-DCs could induce HLA-A2-restricted and the TAA-derived SPs-specific CTLs *in vitro*. This technology may well provide universally applicable dendritic cells of which HLA class I molecules expressed on the cell surface are compatible to the recipient's (patients') HLA-class I molecules and useful for iPS-DC-based cancer immunotherapy.

restricted and the TAA-derived SPs-specific CTLs *in vitro*[19]. We are planning to develop a cancer immunotherapy by using these TAA-derived peptides and human iPS-DCs (Figure 3).

In conclusion, the CDCA1-, KIF20A- and LY6K-derived LPs provide a tool for propagation of tumor-specific Th1 cells and CTLs. Our studies support a possible clinical application of LPs-based cancer immunotherapy. We also propose a usefulness of HLA class I compatible TAP-deficient human iPS cell-derived DC for the future cell-based cancer immunotherapy.

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Specialty and Present Interests:
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DENDRITIC CELL VACCINATION AGAINST CANCER: FROM PRECLINICAL RESEARCH TO PHASE III TRIAL

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Cancer-specific T cells are often present in patients suffering from so-called immunogenic tumors with cutaneous melanoma being the best studied example. The emergence of such T cells, likely induced by dendritic cells (DC) that have taken up dying tumor cells and received maturation stimuli sufficient to let them switch from the tolerogenic to the immunogenic mode, cannot have a significant impact on the clinical course at the metastatic stage simply because the T cells are made useless by various immunosuppressive mechanisms in the tumor microenvironment. Checkpoint blockade can unleash these T cells, and has yielded unequivocal clinical results even in initial trials thus being primarily responsible for cancer immunotherapy being selected as the scientific breakthrough of the year 2013 and for convincing even the most conservative chemotherapy-oriented oncologists that T cell-mediated immunotherapy of cancer will have a bright future. The question now is whether cancer vaccines will finally play a role or not in melanoma or other tumors. Given the above scenario in retrospect it is not surprising that vaccines even if appropriately immunogenic (i.e. inducing sufficient amounts not only of functionally competent $CD4^+$ but also $CD8^+$ tumor-specific T cells) could produce clinical effects only in subsets of metastatic melanoma patients. It is now also understandable, that vaccines that probably have not induced tumor-specific $CD8^+$ T cells or primarily resulted in $CD4^+$ T cell responses were totally disappointing.

We like other academic groups continue to study cancer vaccines addressing the following hypotheses: 1.) in tumors such as melanoma, which we are studying, it will be rewarding to combine vaccination with checkpoint blockers (for enhanced efficacy and less side effects, as already suggested by the results of a recent pilot trial performed by the K.

Thieleman's group) or treat in an adjuvant or neoadjuvant (e.g. regressing tumors in response to BRAF inhibitors) setting with minimal tumor load and less immunosuppression for less relapse and prolonged survival (as for example suggested by our own trial results and those of Carl Figdor's group); 2.) in *non*-immunogenic tumors appropriate cancer vaccines might be critical in the metastatic and even more so in the adjuvant setting to induce tumor-specific immunity which (only) then can be pushed by checkpoint blockade or other interventions; 3.) it will be critical to develop highly immunogenic vaccine strategies, also capable of driving memory T cell differentiation, and 4.) capable of inducing T cells specific for mutated passenger and driver mutations. Seminal work by the T. Boon group has shown that although Cancer Testis (CT) antigens can function as rejection antigen a second wave of endogenously induced ("endogenous vaccination") T cells directed to tumor-specific mutations seems critical in regressing individual metastases ([1] and literature cited therein). This observation not only for the first time proved that regressions occur because of vaccination and not despite vaccination, but also revealed the relevance of mutation-specific T cells at the effector arm. The importance of such T cells has recently been supported by refined immunomonitoring data in melanoma patients with regressions upon adoptive T cell transfer or ipilimumab treatment by S. A. Rosenberg and T. Schumacher, respectively[2,3]; and 5.) the hypothesis that vaccines even if clinically effective will reach clinical practice only if their production is feasible, reproducible and cost-effective. An illustrative example for this logistical aspect is Dendreon's Provenge ™ vaccine, approved by the FDA in 2010 as first active cancer immunotherapy ever. It is a DC-based vaccine which was simply too complicated to manufacture requiring one apheresis for each vaccine shot so that it was also for this reason commercially not viable finally now resulting in bankruptcy of the company.

We have been working for many years to optimize dendritic cell (DC) vaccination, i.e. the adoptive transfer of antigen-laden DC as vaccine strategy. We use monocyte-derived DC, whereby immature DC are generated by exposing blood monocytes to GM-CSF + IL-4 over 6 days followed by addition of an inflammatory cocktail (IL-1beta + IL-6 + TNF alpha + PGE_2) to reproducibly yield very homogenous populations of mature DC that can be loaded with antigen by adding exogenous peptides or by transfection of mRNA after rather than before maturation using a patented electroporation approach[4]. Monocytes are elutriated from aphereses directly into culture bags for DC generation, and this process is (already) semi-closed, highly reproducible and effective (yield 400-600 million mature DC), cost-effective (altogether 4,000 Euro per vaccine shot production cost), and GMP production has been approved already for phase III trials according to the rules of the EMA every company would have to follow in a pivotal trial. In cooperation with Miltenyi Biotec Inc. their Prodigy cell processing system is currently adapted to avoid also the last open

step, namely electroporation, to allow a semiautomated highly standardized DC vaccine production. An automated closed system is also under development by Argos Therapeutics Inc. The message is that production at least of monocyte-derived DC will no longer pose an obstacle for large pivotal trials and commercialization, the only critical issue is to make the DC vaccine effective.

Our monocyte-derived DC vaccines have been tested over the years in several trials, and I have reported results of two so far unpublished ones, one using peptide-loaded first generation and another one employing RNA-transfected second generation DC vaccines, and encompassing CT as well as melanocyte differentiation antigens. These trials are illustrative in that advanced metastatic cutaneous melanoma patients were vaccinated and followed up for years before the modern checkpoint or BRAF inhibitor therapies became available. The vaccines were uniformly immunogenic, inducing IFN gamma producing $CD4^+$ T cells (often strongly *ex vivo* detecable) and polyfuntional $CD8^+$ T cells (rarely clearly *ex vivo* detectable indicating room for improvement) that lysed target cells in part with high affinity. Of note was the fact that overall survival was definitely comparable to what has been observed in non-resectable metastatic melanoma patients upon second line anti-CTL-A4 (ipilimumab) therapy with 25% of stage IV (70% M1c) melanoma patients living 50 months after start of DC vaccination and formation of a "shoulder" of long-term survivors. As expected based on what we know today there was no clear correlation between detection of vaccine-induced T cells, even after we have recently concluded more extensive immunomonitoring studies. Strikingly, there was, however, a statistically significant correlation between emerging transient eosinophilia after the first series of vaccine shots and later long-term survival (defined as beyond at least 2 years). The relevance and mechanism of the eosinophilia observed is unclear at present but it is noteworthy that eosinophilia is a well-known phenomenon after IL-2 application, is observed in the course of GVHD, and appears to evolve also in ipilimumab-treated patients with prolonged survival. The phenomenon possibly indicates successful *in vivo* effects of DC on the patient's immune system (strong T cell activation, but perhaps also changes of the innate immune system) with eosinophilia being just a simple biomarker readout. Within an EU consortium we (Carl Figdor's and our DC vaccine group in cooperation with Duccio Cavalieri as systems biologist) have observed that certain transcriptome changes occur in PBMC after the first series of DC vaccines in patients who later show prolonged survival supporting such an interpretation (manuscript in preparation). To enhance the stimulatory activity of our DC we introduced a highly engineered CD40L mRNA (obtained from Argos Therapeutics Inc.) into our cocktail-matured DC while Argos uses another maturation method[5]. These CD40L-DC like the so-called Tri-Mix DC (generated by transfecting immature DC with mRNA coding for constitutively active TLR-4, CD70 and non-

engineered CD40L mRNA) displayed an enhanced T cell stimulatory capacity *in vitro*. When we explored the DC transfected with RNA coding for defined antigens (with or without mRNA coding for CD40L or TriMix) in various cohorts we found that the intravenous route, we had already tested in our first in man trial, appeared to produce equivalent or better results than the intracutaneous route (as also found by Kris Thieleman's group[6]) so that this is now our preferred administration route. Of interest is also that i.v. administration resulted in higher eosinophilia and constitutional symptoms (including elevated body temperature first observed about 8 hours after the second or third but not the first DC vaccination), both preferably occurring in patients which later turn out to survive longer.

We use such second generation DC transfected with PCR amplified total tumor mRNA now in an ongoing phase III trial for adjuvant vaccination after resection or destruction of so-called monosomy 3 uveal melanomas which in contrast to uveal melanomas displaying both chromosomes 3 metastasize in the large majority of patients. The DC type we use has already been proven capable of inducing *in vivo* de *novo* responses to mutated (but not wild type) GNAQ using overlapping 9mer peptide libraries as readout.

In an effort to further improve our DC vaccine we have studied in more detail how helper T cells help human DC to stimulate $CD8^+$ T cells[7]. Helper T cells in seminal studies have been shown to license DC for $CD8^+$ T cell induction notably by expressing CD40L and delivering a stimulus to CD40 expressing DC. Often this signal has been studied using immature DC which were then induced to mature hereby switching DC into their immunogenic mode and “licensing” them to prime $CD8^+$ T cells. We have now found that mature DC albeit they prime well do not imprint a "memory type" differentiation program in $CD8^+$ T cells unless helper T cells, $CD8^+$ T cells and DC stay together forming a tri-cellular complex[7]. Only then antigen-specific $CD8^+$ T cells are primed which upon subsequent antigen-specific restimulation prove highly proliferate and functionally competent (enhanced lytic capability and cytokine production). If helper T cells are taken away after the initial contact with and activation by the DC (so-called sequential model) priming is similar but induced antigen-specific $CD8^+$ T cells do not proliferate well upon subsequent restimulation. The message here is that while maturation of DC is irreversible the subsequent activation stimulus delivered via antigen-specifically stimulated helper T cells to DC is transient. Importantly we found that our effort to imitate "T helped" DC by transfecting highly engineered CD40L mRNA into cocktail-matured DC was just like Tri-Mix DC not fully successful (see Figure 1) in that the activated state of the DC was transient lasting only a few hours. We, therefore, next explored expression of constitutively active stabilized IKK[8] to directly activate NF-B over a prolonged duration which resulted in a third generation of "Super Designer DC" capable of inducing highly proliferative $CD8^+$ T

cells without need for antigen-specific help (see Figure 1). These DC are the ones we finally will use in future trials. In preclinical studies we have shown that they can reliably prime $CD8^+$ T cells of high quality and proliferative potential including T cells recognizing peptides derived from driver mutations such as NRAS. These DC which can soon be produced using a semi-automated closed system thus represent a universal yet cost-effective vaccine platform suitable for addressing the hypotheses and open questions mentioned above.

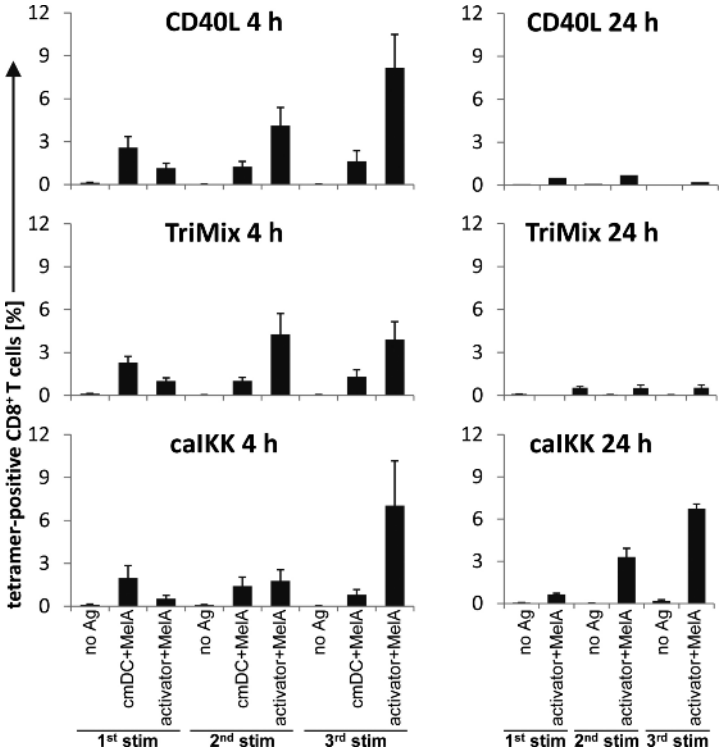

Figure 1 Monocyte-derived DC were cytokine cocktail-matured and electroporated with MelanA RNA ($cmDC^+$ MelA) or were not loaded with antigen (no Ag). In addition, DC were electroporated with RNA encoding activators in addition to MelanA ($activator^+$MelA). CD40L, TriMix, and constitutively active IKK (caIKK) were used as activators. TriMix is a mix of constitutively active TLR4, CD40L, and CD70, which was electroporated into immature DC, CD40L and caIKK were electroporated into cocktail-matured DC. Four hours (left hand side) or 24 h (right hand side) after electroporation, these DC were used to stimulate autologous $CD8^+$ T cells in a 1:10 ratio. One week after stimulation, part the T cells were re-stimulated with cryopreserved DC similar to the first stimulation. The percentage of MelanA-specific T cells was analyzed by MHC-tetramer staining after priming (1^{st} stimulation) and two re-stimulations (2^{nd} and 3^{rd} stimulation). The average values of 4 (CD40L 4 h), 2 (CD40L 24h), 7 (TriMix 4 h), 9 (Trimix 24 h), 5 (caIKK 4 h), and 3 (caIKK 24h) independent experiments with SEM are shown. (part of the data was published in: Pfeiffer et al. Eur J Immunol. 2014 Aug 6. doi: 10.1002/eji.201344417)

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IMMUNOTHERAPY IN ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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Allogeneic hematopoietic cell transplantation (HCT) is a curative therapy for patients with complex hematological malignancy and one of the first clinical settings demonstrating that immune effects can eradicate cancer. In this setting donor derived T cells are critical for graft vs tumor (GVT) effects yet can also result in graft vs host disease (GVHD). Developing clinical strategies to reduce GVHD while maintaining GVT are key to improving outcomes. We have explored several different strategies including the timed addition of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}) as well as *ex vivo* expanded cytokine induced killer (CIK) cells initially in animal models with extension to the clinic.

We have utilized bioluminescence imaging (BLI) to evaluate the fate of adoptively transferred T cells and have developed luciferase (luc) expressing transgenic animals which allow for the isolation of different cell populations that can be followed after the administration of luciferin the substrate of luc. Utilizing these animal models we have identified that allogeneic T cells rapidly infiltrate nodal sites and begin to proliferate resulting in the upregulation of key molecules that allow for entry into GVHD target tissues such as the skin, gastrointestinal tract and liver. Once activated GVHD is difficult to control especially once target tissue infiltration has occurred. A number of different strategies have been explored including the use of T_{reg} and CIK cells. T_{reg} have potently resulted in the control of GVHD mainly through reducing the proliferation of the alloreactive T cells[1)]. Yet T_{reg} do not appear to interfere with GVT responses in these animal models due to the activity of the alloreactive conventional $CD4^+$ and $CD8^+$ T (T_{con}) cells that can recognize foreign cells, in this case, cancer cells. Limitation of cell proliferation which controls GVHD does not appear to limit GVT effects, especially when adequate numbers of

T_{con} are administered and the tumor burden is low[2]. Recently, a novel approach of using the monoclonal antibody 4C12 which reacts with DR5, a TNF superfamily member has resulted in dramatic expansion of donor T_{reg} and reduction in GVHD risk when donors are treated with a single injection of antibody. Further another population of donor derived invariant natural killer T (iNKT) cells have potently suppressed GVHD in these murine models[3]. Interestingly, the iNKT cells require donor T_{reg} for optimal function.

The use of donor derived T_{reg} have been translated to the clinic with excellent results both by the groups from the University of Perugia and University of Minnesota. These initial studies demonstrate that T_{reg} can be isolated from donors and in the latter study expanded *ex vivo* with apparent reductions in GVHD risk[4,5]. Interestingly, in an update from the University of Perugia, low relapse rates have been observed following adoptive transfer of T_{reg} and T_{con}[6].

Another strategy to enhance the GVT effects of allogeneic transplantation involve the use of cellular populations that have limited capacity for GVHD yet are capable or recognizing and eliminating malignant cells. Cytokine induced killer (CIK) cells are an expanded cell population prepared by the timed addition of interferon-γ, CD3 stimulation with monoclonal antibodies such as OKT3 and interleukin-2. Following culture for 14-21 days there is marked expansion primarily of T cells with co-expression of NK markers such as CD56 and NKG2D. CIK cells have been studied in both murine and human models. In murine models, CIK cells have been shown to have potent anti-tumor activity and in the allogeneic setting, do not result in graft-versus-host disease (GVHD)[7,8]. Using bioluminescent imaging CIK cells traffic to tumor sites primarily due to the inflammatory nature of the tumor and chemokine gradient[9,10]. Once at the tumor site, the cells are capable of recognizing tumor cells primarily through an NKG2D mediated mechanism, although other cytotoxic mechanisms are operative. CIK cells have been utilized in a variety of different clinical settings both in solid tumors, as well as hematologic malignancies. Our group has been particularly interested in the setting of allogeneic hematopoietic cell transplantation and it is well recognized that graft-versus-tumor (GVT) effects are critical. We have expanded CIK cells from both related and unrelated donors and demonstrated that this is a feasible approach, responses occur and GVHD is not exacerbated[11]. We have developed a preparative regimen using total lymphoid irradiation and anti-thymocyte globulin (TLI/ATG) which is safe and results in a low incidence of GVHD, yet relapse remains a persistent problem[12]. We have explored the use of TLI/ATG in a series of patients with myelodysplastic syndrome (MDS)[13] using this as a model system for assessing the overall benefit of allogeneic transplantation and the planned addition of CIK cells. With TLI/ATG conditioning patients with MDS have an ~40% two year survival with ~50% risk of relapse with a low incidence of GVHD and transplant-related mortality

(TRM). Due to the high risk of relapse, we have added CIK cells in 25 patients at day+21 similarly selected. Up to $2x10^8$ CIK cells/kg can be obtained and these cells do not result in exacerbation of GVHD or TRM. Relapse risk appears to be reduced, as well as improvement in overall survival. Another approach actively under investigation is the use of CIK cells to deliver other anti-tumor biotherapies. We have focused on the use of CIK cells and oncolytic viruses since oncolytic viruses have been shown to result in anti-tumor effects, yet require injection directly into the tumor site. Upon infection with mutant strains of vaccinia virus there is an eclipse period of 48-72 hours that is advantageous since this is the period of time it takes for CIK cells to migrate to the tumor sites. Upon release of the virus there is direct cytotoxicity to the tumor cells associated with further upregulation of NKG2D ligand expression. This strategy has been effective in a variety of different murine models and is being readied for clinical translation[14]. In sum, CIK cells could be useful to develop alternative approaches to cell mediated anti-tumor responses.

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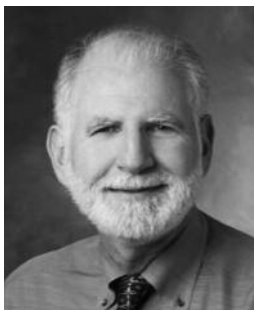

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Specialty and Present Interest:
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CISH ATTENUATES PROXIMAL TCR SIGNALLING AND ITS DELETION ENHANCES TUMOR DESTRUCTION

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In his Nobel lecture in 1980, George Snell described how tumor transplantation experiments led him to the discovery of MHC. Zinkernagel and Dougherty, who won the Nobel Prize in 1996, continued to provide insight into the structures recognized by T cells. These fundamental discoveries have laid the groundwork for the molecular definition of tumor rejection antigens, including those derived from mutated antigens, and this work continues at the present time.

Although the target structures that are recognized by anti-tumor T cells are of paramount importance, it has become clear that the mere recognition of antigen/MHC structures is insufficient to destroy tumor cells. Molecular elements downstream of TCR recognition can truncate T cell functions, although these elements remain incompletely elucidated. One class of potentially critical negative regulators of immune cell function are the Suppressors of Cytokine Signalling (SOCS), a family of eight proteins (SOCS 1-7, and the founding member Cish) that share a central SH2 domain and a 'SOCS box' critical to their functions as E3 ligases (see Figure 1).

We characterized a *Cish*$^{-/-}$ mouse and found it to be healthy and breed well. *Cish*$^{-/-}$mice were more resistant to infectious challenge with vaccinia virus. Deletion of *Cish* resulted in better survival, viral clearance and increased numbers of CD8^{+}IFNγ^{+} T cells. Gene-expression profiling revealed that *Cish*-deficient CD8^{+} T cells more robustly expressed genes downstream of TCR-signalling indicating that Cish was likely acting very proximal to the TCR signalling, to truncate the signalling event itself. Biochemical analysis of the signalling machinery yielded the clear result that Cish was physically interacting with phospholipase Cγ1 (PLCγ1), resulting in its degradation. Activated PLCγ1 converts

phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol, consequently potentiating calcium flux, Protein Kinase C activation and NFAT and NFκB transcriptional activity, properties found to be enhanced in *Cish*$^{-/-}$ mice. We observed that the adoptive transfer of *Cish*$^{-/-}$ tumour-reactive CD8^{+} T cells resulted in increased T cell expansion, pronounced and sustained tumour regression, and enhanced *ex vivo* target-reactivity. Thus, *Cish* plays a significant role in CD8^{+} T cell immunity by attenuating proximal TCR signalling and immunity to pathogens and cancer and that the deletion of *Cish* unleashed PLCγ1 activation and durability (Figure 2). This approach may be useful in the augmentation of cancer regression.

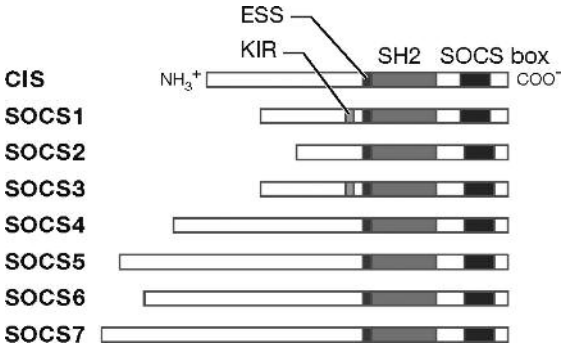

Figure 1 CIS is the founding member of the SOCS family of evolutionarily conserved proteins. Abbreviations – SOCS: Suppressors of Cytokine Signalling; KIR: Kinase inhibitory region; ESS: Extended SH2 domain. Adapted from Palmer DC and Restifo NP reference 1.

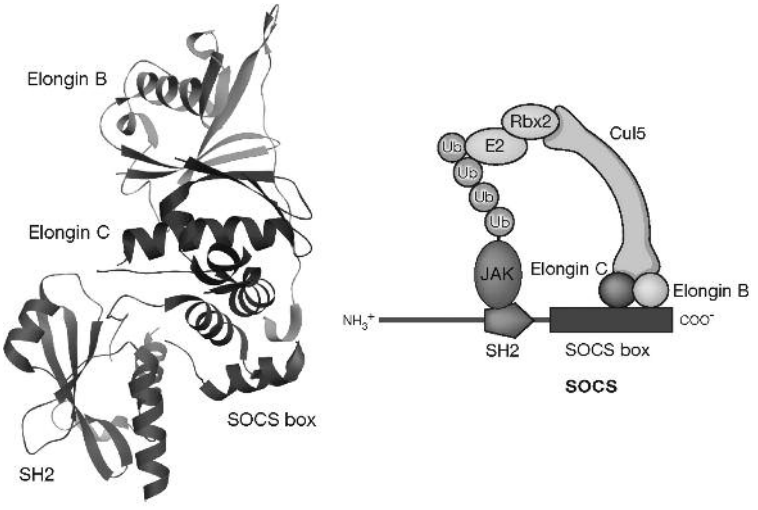

Figure 2 SOCS molecules form E3 ligases, targeting proteins for degradation. Adapted from Palmer DC and Restifo NP reference 1.

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Specialty and Present Interest:
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ENGINEERING T CELL RESPONSES TO EFFECTIVELY TARGET TUMORS: INSIGHTS FROM PRECLINICAL MODELS AND CLINICAL RESULTS

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Effective cellular therapy for human malignancies requires first identifying and validating an appropriate antigenic target, and then establishing in each patient a tumor-reactive T cell response of high avidity and high magnitude that is safe and can infiltrate and retain function in the tumor microenvironment. We have been exploring in preclinical models and clinical trials methods to reproducibly provide such responses by transfer of genetically engineered T cells. We examined purified human leukemic stem cells for over-expression of genes in comparison to purified human hematopoietic stem cells as well as normal somatic tissues. Our analysis revealed that WT1, a gene known to be associated with promoting leukemic transformation, and demonstrated to be an immunogenic target in human cancers by the Sugiyama lab, is expressed in comparative abundance in human leukemic stem cells[1]. Preclinical studies were then performed in a mouse model, and revealed that CD8 T cells specific for this oncogene with even higher avidity than can be detected in normal repertoires could be safely administered, with no evidence of toxicity to the normal tissues known to express low but detectable levels of WT1[2]. These studies led to performing our initial clinical trial in patients with leukemia who relapsed after hematopoietic cell transplant (HCT), a group of patients with a very poor prognosis. For this trial, we transferred WT1-specific CD8 T cells clones isolated and expanded *in vitro* from the HCT donor, and demonstrated that such T cells were safe, mediated *in vivo* anti-leukemic activity, and were associated with maintenance of long-term remissions in some patients[3]. However, generating sufficient numbers of WT1-specific CD8 T cells with high avidity for the target in each patient represented a substantive problem. To overcome this obstacle and create a standardized reagent for treatment of patients whose tumor expresses

the target antigen and shares an MHC restricting allele, we pursued methods to genetically engineer patient T cells to acquire high avidity for the tumor target by introducing and achieving high level expression of the genes encoding the V and V genes of a TCR demonstrated to have high affinity for the target epitope (Figure 1). This required developing strategies to overcome the potential problem of mispairing of the introduced TCR chains with the endogenous TCR chains[4,5], and incorporating changes in the gene construct such as codon optimization to enhance expression. We then screened a large number of normal repertoires for the presence of high avidity WT1-specific CD8 T cells, and selected the T cell clone expressing the highest affinity TCR. We have now have now initiated a trial in which this high affinity, WT1-specific, A2-restricted TCR is being introduced into patient CD8 T cells with a lentiviral vector and the transduced cells are being infused into the patient. The early results from this trial appear promising in terms of both evidence of antileukemic activity and the capacity for the transferred cells to persist in the patients, and we plan to begin very shortly another trial in patients with non-small cell lung cancer (NSCLC) utilizing this same TCR, as WT1 is also commonly overexpressed in NSCLC.

However, isolating high affinity TCRs for many tumor antigens that are also normal self-antigens may not be readily achieved from normal repertoires. Therefore, we have developed strategies to enhance the affinity of isolated TCRs with retention of specificity, including both saturation mutagenesis of CDR3 regions[2] and an *in vitro* thymic selection

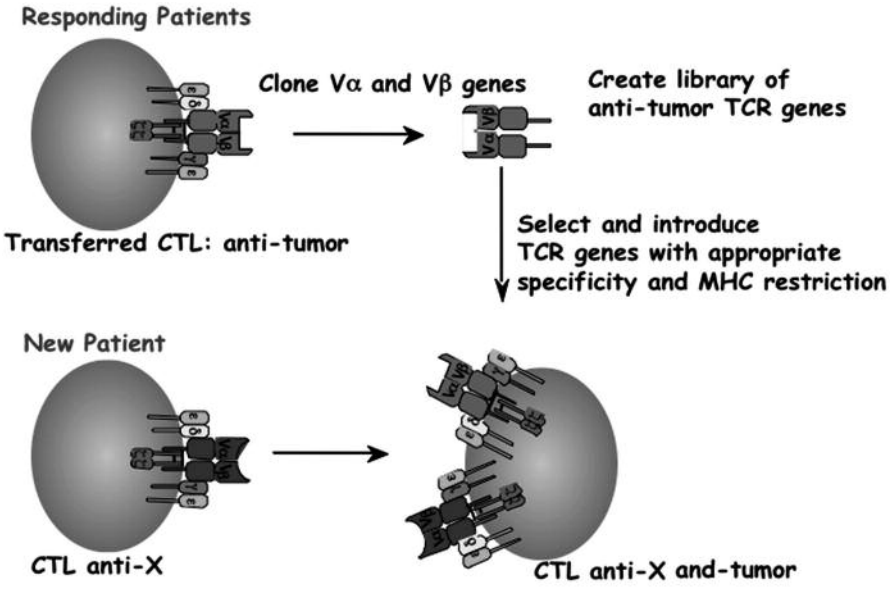

Figure 1 Impart Target Specificity by Introduction of High Affinity TCR Genes

system that allows for capture of a more diverse set of high affinity specific TCR genes during TCR gene rearrangement. The strategies being pursued are designed to minimize the risk of off-target toxicity from promiscuous epitope recognition, and we are further using bioinformatics as well as modeling in the mouse to decrease the potential for such off-target toxicity.

Unfortunately, providing a high avidity T cell response does not necessarily result in tumor eradication, as there are other substantive obstacles that can preclude even a T cell expressing a high affinity TCR from being effective. These impediments include the development of T cell dysfunction by cell intrinsic and extrinsic pathways[6], particularly within the tumor microenvironment[7], and we are using genetically engineered mouse models to elucidate the cellular and molecular pathways[8] that need to be modulated to achieve meaningful therapeutic benefit in a variety of solid tumor settings, including pancreatic[7] and ovarian cancer. Our preclinical therapy studies, particularly in the pancreatic cancer model, already appear very promising, and we plan to use the insights derived from these studies to initiate within the next 1-2 years clinical trials in human pancreatic and ovarian cancers. The genetically-engineered mouse models we are using are making it possible to assess T cell therapy targeting a tumor antigen that is newly expressed in a solid tumor that develops "spontaneously" as a consequence of regulated tissue-specific expression of an oncogene and for tumor antigens also detected at low levels in normal tissues. These studies are revealing the importance of not only cell extrinsic mechanisms of regulation and dysfunction that render T cells unresponsive, particularly via inhibitory pathways commonly operative within the tumor microenvironment that interfere with an effector response such as the accumulation of regulatory CD4 T cells (T_{reg}), myeloid derived suppressor cells (MDSC), and tumor-associated macrophages (TAM), but also the cell intrinsic mechanisms that ultimately can epigenetically dictate maintenance of a tolerant or non-responsive state. These cumulative mechanisms highlight the difficulties eliciting and/or sustaining responses to tumor antigens. Therefore, we are using the models to evaluate strategies to sustain function and anti-tumor activity by modulating, selecting, or genetically modifying T cells to be resistant to obstacles that prevent tumor eradication or re-establish a tolerant state. As different tumor types exhibit unique characteristics and are capable of engaging distinct pathways, improved understanding of the immunobiology of the particular tumor to be treated will likely prove essential for designing effective therapies. However, the relatively straightforward means to use synthetic biology to genetically engineer T cells to acquire novel capacities to overcome inhibitory signals and function in the tumor microenvironment suggests that cancer therapy with engineered T cells will likely find an increasing role in the treatment of human cancers.

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Specialty and Present Interest:
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ADOPTIVE IMMUNOTHERAPY WITH TCR GENE-TRANSDUCED LYMPHOCYTES

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Engineering the antigen receptor gene in patients' lymphocytes is one promising strategy to create antigen-specific lymphocytes without senescent phenotypes. The strategy provides an opportunity to extend the application of adoptive T cell therapy for patients with various types of cancer (Figure 1). However, this concept has not been well tested in patients with epithelial cancer or hematological malignancy.

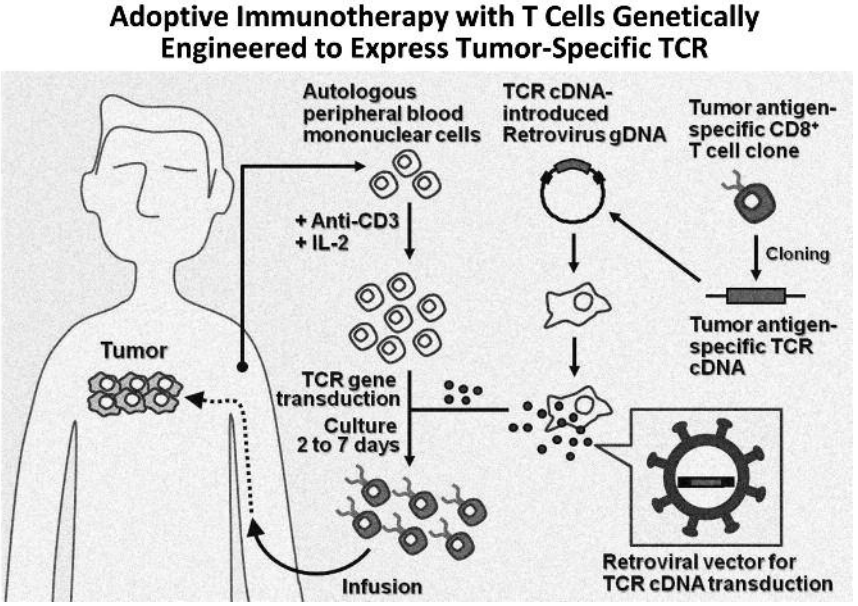

Figure 1

We completed a phase I clinical trial of TCR gene therapy targeting MAGE-A4 to treat esophageal cancer patients without lympho-depleting pre-conditioning (Figure 2). MAGE-A4-specific TCR α and β chains were isolated from a human T cell clone that recognizes MAGE-A4 $_{143\text{-}151}$ peptide in an HLA-A*24:02 restricted manner[1]. This T cell clone did not show any cross reactivity to the peptides with a homology to the MAGE-A4 epitope. A retroviral vector that encodes these TCR chains without any artificial modification was constructed[2,3]. The trial was designed as a cell-dose escalation consisting of three cohorts, $2x10^8$, $1x10^9$ and $5x10^9$ cells/patient. The treatment was tolerable with no adverse events associated with transferred cells. In all patients, the transferred lymphocytes were detected in their peripheral blood in a dose-dependent manner during the first 14 days. The infused cells persisted more than 5 months after the transfer and sustained the reactivity to the antigen-expressing tumor cells. Three patients showed SD or long tumor free status. These results suggest that this approach may extend the availability of adoptive T cell therapy for epithelial cancer patients by providing tumor-reactive and long surviving lymphocytes reducing the risk of intensive pre-treatments.

We have developed a unique retroviral vector that encodes tumor-specific TCR and siRNAs that specifically down-regulate endogenous TCR in lymphocytes (Figure 3). This vector could enhance the expression of the transduced TCR and minimize the risk to create mis-paired TCR[4,5].

Utilizing this novel "siTCR vector", we have started a phase I trial of WT1-targeted TCR gene therapy for patients with leukemia/MDS[6], and also of MAGE-A4 target TCR gene therapy for patients preconditioned with cyclophosphamide and fludarabine.

Development of TCR-gene therapy with allogeneic T cells utilizing siTCR vector silencing endogenous TCR expression

An alternative use of this novel siTCR vector is its application for the use of allogeneic lymphocytes for infusion. Down-regulation of expression of endogenous TCR genes may facilitate to avoid GVHD (graft versus host disease) when T cells transduced with tumor specific TCR with this vector are infused to allogeneic recipients. We explored the possibility by analyzing whether siTCR gene transduction lead to reduction of endogenous TCR expression. In fact, thus treated lymphocytes showed apparent reduction of expression of endogenous TCR with dominant reduction of proliferation when stimulated by allogeneic lymphocytes in mixed lymphocytes culture test. When these receptor gene modified human lymphocytes were infused into immune deficient NOG mice, reduced or essentially no GVHD against xenogeneic hosts of NOG mice was observed while infusion of control lymphocytes of the same donor without siTCR gene transduction resulted in severe GVHD. The use of siTCR transduced allogeneic donor derived lymphocytes in

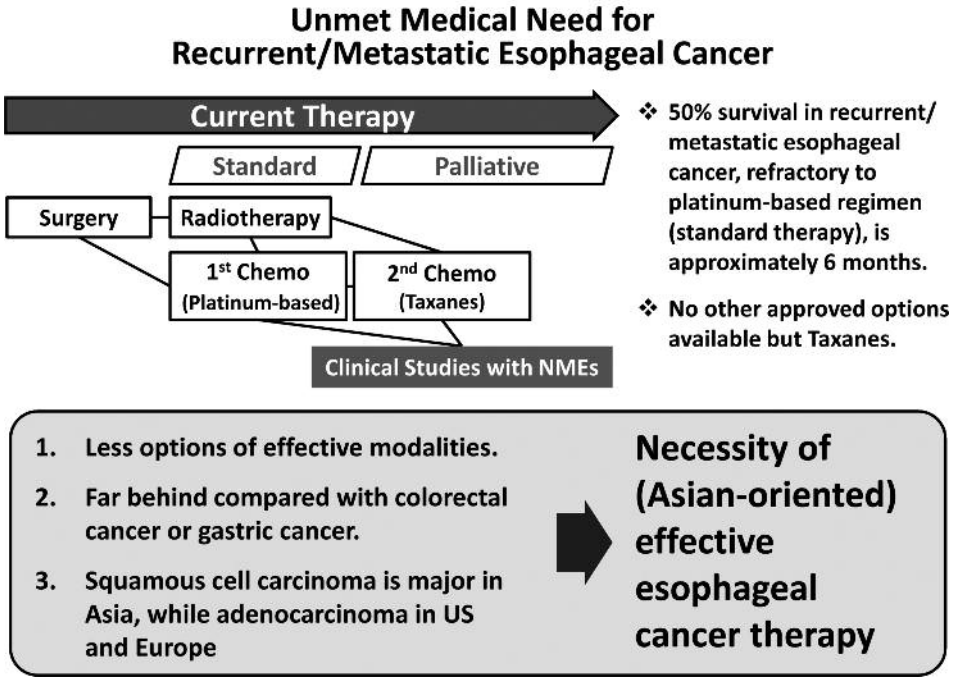

Figure 2

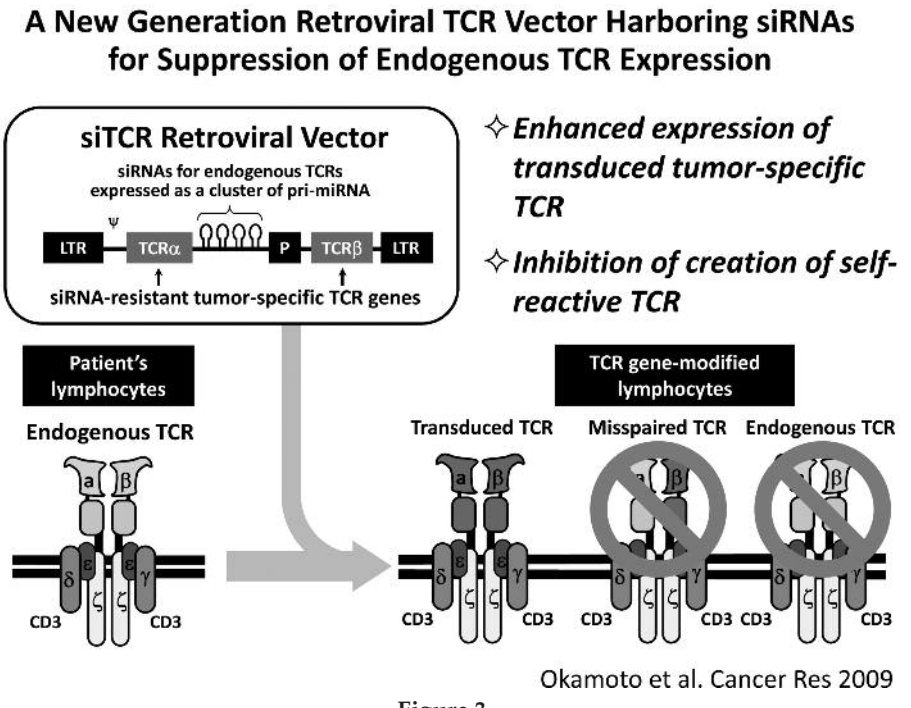

Figure 3

combination with hematopoietic stem cell transplantation is a reasonable next step.

CAR (Chimeric antigen receptor)gene-transduced T cells specific for WT-1 derived peptides with MHC

Another major approach of antigen receptor gene modified lymphocytes is CAR gene transduced lymphocytes. For this, strict tumor specificity of targeted antigen molecules is essential. Since there is no readily available cell surface antigens recognized by antibodies with strict sense, we focused our effort for raising antibodies against peptide-MHC complexes expressed on tumor cells. A number of intracellular tumor specific molecules are known to be expressed on cell surface as peptide-MHC complexes (Figure 4). Screening with phage libraries of human immunoglobulin resulted in obtaining antibodies reactive with a complex of WT-1 derived peptide with HLA-A2402. The scFv of thus obtained antibodies allowed us to construct retrovirus vectors expressing CAR in combination with signal transduction element of T cells. CAR expressing lymphocytes recognize T2A24 cells pulsed with cognate WT-1 derive peptides in the context of HLA-A2402. They also react with a number of cell lines co-expressing WT-1 derived peptides and HLA-A2402 indicating their recognition of the complexes derived from endogenous WT-1 molecules. A manner of WT-1 derived peptide recognition by these CAR T cells was analyzed in comparison with readily available WT-1 TCR gene transduced lymphocytes recognizing

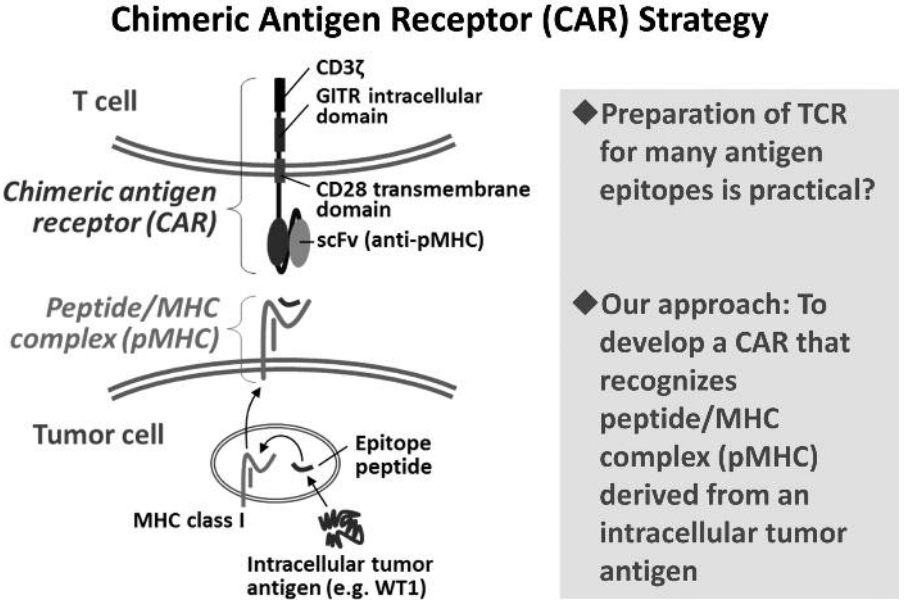

Figure 4

the same peptides. CAR expressing T lymphocytes showed apparently different peptide recognition pattern from TCR gene transduced T cells. However, both of them still demonstrated clear WT-1 derived peptide specificity, showing no reactivity with any other peptides of sequence similarity in the range of our study. These results may indicate a possible use of CAR T cells for peptide MHC complexes may provide an alternative approach for adoptive cell therapy.

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Specialty and Present Interest:
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DESIGNING CAR T CELLS FOR CANCER THERAPY

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It is now realized that T cell immune surveillance of cancer occurs in many cases. One approach that has now been developed to deal with tolerance to tumors has been checkpoint blockade, which can enable the natural immune response to respond to antigens encountered in the tumor microenvironment. In this seminar I will outline recent results in the area of synthetic biology, which uses engineered T cells to overcome immune tolerance. There are three types of T cell therapy that are being actively tested in the clinic[1]. In one case tumor infiltrating lymphocytes are obtained from surgical biopsies. This therapy is most advanced in melanoma, and trials for patients with metastatic melanoma are currently underway with phase 3 trials in Europe. Gene transfer technologies can also be used to produce engineered T cell therapies. One approach is a chimeric antigen receptor or CAR T cell approach. Another is the use of engineered T cells that express T cell receptors of known specificity and affinity.

There are number of considerations for successful T cell therapy with adoptively transferred T cells. It is necessary to have an adequate number of T cells in order to eradicate large numbers of tumor cells. If a kilogram of tumor represents approximately $1x10^{12}$ T cell tumor cells, then it is likely that a similar number of T cells will be required for an equivalent effector to target ratio *in vivo*. The failure to achieve a critical mass of T cells numerically may explain many previous trials with disappointing results following therapeutic vaccines. There are two potential solutions to overcome inadequate effector T cell function in cancer patients with adopted cell transfer therapy. On the one hand it is possible to infuse very large numbers of T cells, as has been done in tumor infiltrating

lymphocyte therapy[2]. The other approach is to infuse smaller numbers of cells that are programmed to expand extensively in the patient[3,4].

There are three essential factors to consider when developing engineered T cell therapies (Figure 1). One issue under consideration is the nature of the lymphocyte subset to be infused. At present it is not known whether the optimal cell type is a memory cell or a naïve cell. Related to this is the question of the proportion of CD4 helper cells and CTLs. Secondly it is important to consider the aspects of genetic engineering that are used to reprogram the cells[5]. Finally it is necessary to have an optimized approach for the manufacturing of the T cells.

The design of CAR T cells has become increasingly sophisticated (Figure 2). The initial CAR T cells were so-called first-generation CARs and only had signaling from the T cell receptor zeta chain or related molecules. More recently second and third generation CAR T

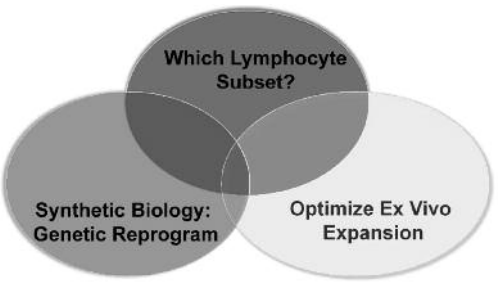

Figure 1 Essential factors for augmenting adoptive cellular immunotherapy.

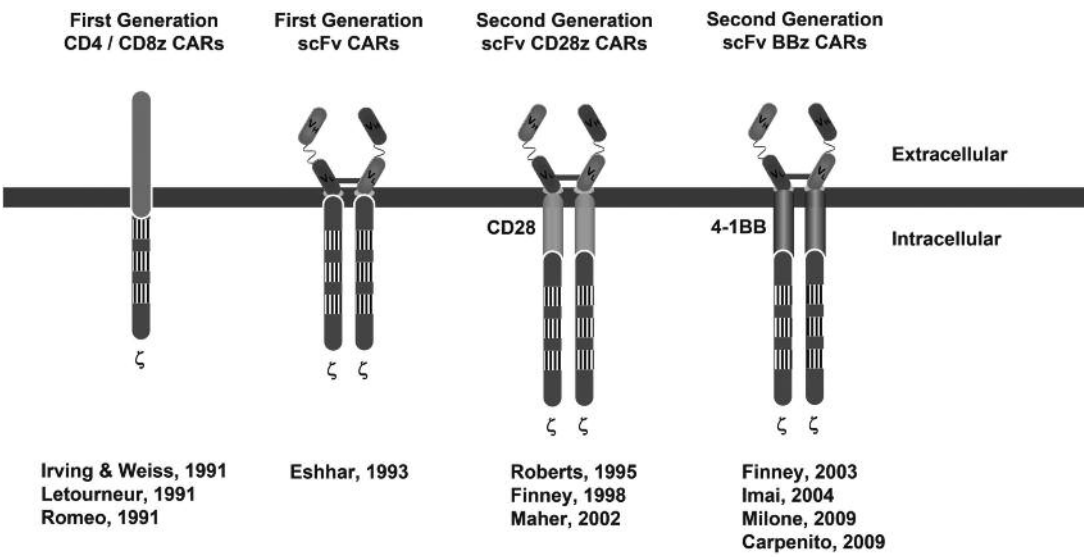

Figure 2 Design of CAR T Cells.

cells have been designed, incorporating progressively more complicated signaling domains. A number of strategies have been used to make CARs more potent or to enhance their proliferative capacity or their cytokine secretion. For example it has been shown that so-called Signal 1 from the Lafferty model[6] can be enhanced by encoding a ubiquitin-resistant linker of activated T cells (LAT)[7]. A number of costimulatory domains have been used to augment Signal 2. These include CD28, and the tumor necrosis family members 4-1BB, Ox40, and CD27, for example. Recent studies have shown that the ICOS signaling domain, a molecule related to CD28, provides CAR T cells with enhanced persistence in preclinical animal models. These studies were initiated by Crystal Paulos and showed that when T cells were activated through their natural ICOS receptor molecule with agonistic antibodies, that this promoted the proliferation of TH 17 cells and T follicular helper cells that had highly potent effector functions (Figure 3). In contrast, if the T cells were costimulated with a CD28 dominant costimulatory signal, then the TH17 cells were restrained in their antitumor activity and inflammatory properties[8]. More recently, Sonia Guedan has demonstrated that when ICOS is expressed in CD4 cells by encoding the cytosolic domain in the CAR endodomain that this leads to enhanced persistence of the CD4 cells and that those cells promote the persistence of CD8 CAR T cells[9]. This latter effect is independent of the costimulatory domain it is expressed in the CD8 CAR T cell.

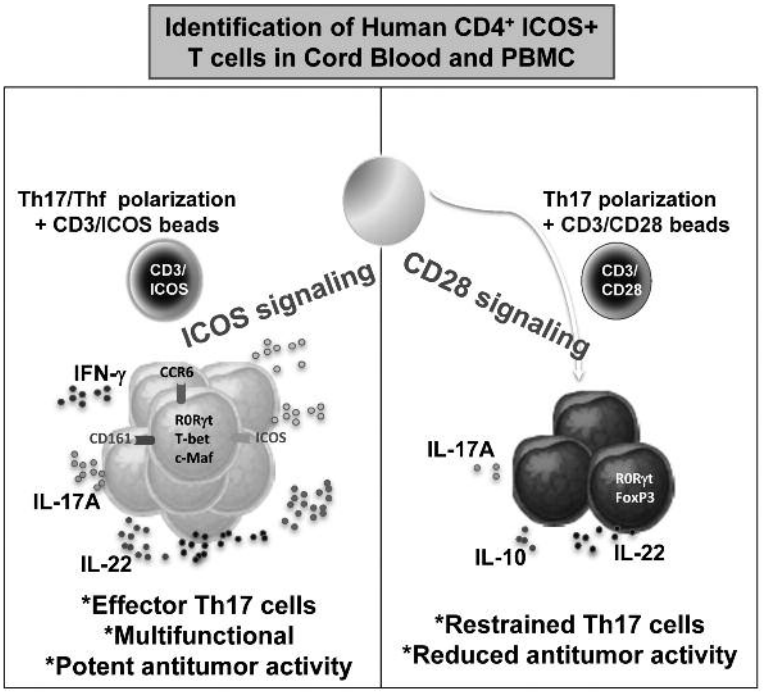

Figure 3 Distinct Cosignaling by CD28 and ICOS: Implications for ACT Therapy

Other studies in our laboratory have recently identified examples of CAR designs that have either a continuous and ligand-independent growth phenotype, or are quiescent in the absence of stimulation by surrogate antigen. Finally, studies by Yangbing Zhou have recently shown that it is possible to increase the therapeutic index of CARs by affinity-tuning of the single chain variable fragments.

Ongoing research is defining the characteristics of CAR design that lead to a continuously active CAR. These T cells are capable of proliferating for months without stimulation or addition of exogenous cytokines. Factors that dictate ligand independent growth of the CAR T cells depend on the particular scFv used, and so far they have only been observed with CD28 endodomains. The growth is independent of exogenous ligands and there is evidence for constitutive signaling through CD28 involving AKT, NF-kappaB and the MAP kinase pathway. The continuous CAR T cells have constitutive secretion of Th1 and Th2 cytokines, and they tend to highly express T-bet, Eomes, GATA3, and Bcl-xL. Transformation not observed, and surprisingly, CAR T cells with the continuous growth phenotype are less effective in pre-clinical tumor models. One implication of this research is that it is important to screen newly designed CAR T cells for constitutive activity.

The initial CAR T cell trials were conducted in patients with HIV, using a first-generation CAR[10]. At the University of Pennsylvania CAR T cell trials in cancer patients targeting CD19 were initiated in 2010[11]. There were three unique aspects of this the CAR, termed CART19, that were used in this trial that have not been tested previously. First a lentiviral vector was used to express the CAR. Secondly, a short duration T cell culture process using antibody-coated beads was used for T cell manufacturing. Thirdly, the CAR employed a TNF family member for co-stimulation, CD1 37, also referred to as 4-1BB. The initial patients that were treated had refractory chronic lymphocytic leukemia[12]. All three of the initial patients responded to the CART19 therapy. The patients had large bulky tumors and it was possible to conclude that each CART19 cell or its progeny through cell division killed more than 1000 tumor cells. As of December 2014 the patients continue to express the CARs and the remissions have been durable in two of the three patients having no evidence of leukemia for more than 4 years. The level of CAR T cell proliferation *in vivo* is a predictive biomarker of clinically beneficial antitumor responses. In more recent studies the CART19 T cell approach has been used to treat patients with acute lymphoblastic leukemia 9ALL). We recently reported the first 30 patients on this phase 1 trial and a striking 90% rate of complete remission was observed in both pediatric and adult cases[13]. Durable B cell aplasia is associated with a low risk of relapse of ALL following CART19 T cell therapy.

There have been a number of toxicities associated with CAR T cell therapy. B cell aplasia is observed in responding patients following therapy with CD19 or CD20 specific CARs.

Tumor lysis syndrome has been observed in many patients, and this is managed as per standard oncology practices and the unique aspect of this is that it may be delayed for 20 to 50 days after infusion of the CAR T cells. Cytokine release syndrome characterized initially with fever occurs in many patients, and this is related to the tumor burden in the patient. Finally, a subset of patients develops macrophage activation syndrome which is characterized in the serum by very high levels of ferritin, C-reactive protein and evidence of coagulopathy. The hallmark of this syndrome are very high levels of IL-6 and interferon gamma in the serum. This syndrome is rapidly reversed with the IL-6 receptor antibody tocilizumab, indicating that this inflammatory disorder is dependent on IL-6 (Figure 4).

In summary, engineered T cells are finally on the path towards widespread use. A number of trials are now underway that should lead to commercial approval in the United States and in Japan.

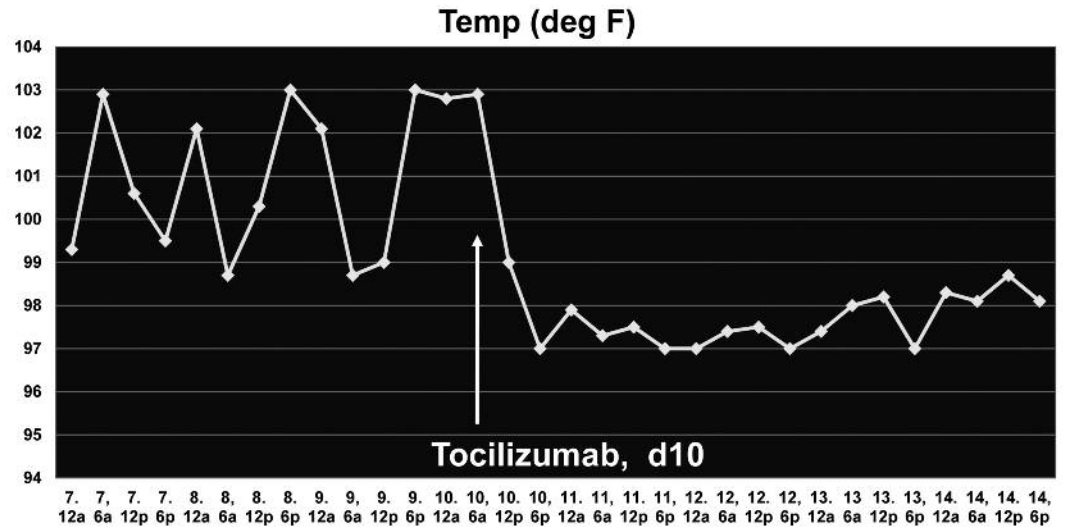

Figure 4 Tocilizumab Anti-Cytokine Therapy for Cytokine Release Syndrome. A patient with leukemia was given tocilizumab on day 10 after CART19 therapy, with immediate resolution of fever.

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CONCLUDING REMARKS

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The 45th Annual Symposium of the Princess Takamatsu Cancer Research Fund was focused on the very timely and important topic of Recent Advances in Cancer Immunotherapy. The recognition of the research area is particularly significant since *Science* declared cancer immunotherapy to be the "Breakthrough of the Year" in 2013. Many of the most international important physicians and scientists in the field traveled to join distinguished colleagues in Japan to discuss the most up to date information in the field. The Symposium indeed covered the most important areas of research progress that have resulted, in recent years, in unprecedented advances for patients using highly innovative approaches.

Much has been learned about the dynamic interaction that exists between the immune system and cancer. It is now well known that T cells can infiltrate tumors early in their development and that important prognostic information can be gained from quantifying the extent and quality of the infiltrate. Further, the identification of the importance of mutational neo-epitopes has validated the importance of the concept of cancer immune surveillance and has given new energy to the quest to develop truly specific cancer vaccines. This area will be complemented by advances in understanding of mechanisms in innate immunity, including the STING pathway. The recognition that tumors are 'seen' by the immune system has allowed for the development of approaches that enhance immune reactivity. One such area is immunologic checkpoint blockade and two classes of checkpoint blocking antibodies have recently gained regulatory approval after demonstration of significant clinical impact. CTLA-4 blockade with ipilimumab was the first intervention ever to improve overall survival in metastatic melanoma and PD-1

blockade has led to impressive clinical responses in both sold tumors and hematologic malignancies. Most recently, combinations of these agents have resulted in rapid and durable regressions in a large subset of patients with melanoma and renal cell carcinoma. Successes such as these have given rise to additional investigations involving agonist agents targeting CD137 alone and in combination with antibodies to antigens on tumor cells. While tumor targeting antibodies have been relatively limited in the past to surface markers on B cell malignancies, Her-2 and EGFR, more current technologies are allowing for the development of antibodies specifically directed to MHC-peptide complexes, allowing for recognition of important intracellular molecules.

For tumors which may not have favorable antigens engendering a baseline immune response, recent developments in the expansion and engineering of T cell products have given way to a variety of approaches which introduce highly activated and specific T cells into the repertoire. The use of expanded tumor infiltrating lymphocytes and T cells bearing transgenic T cell receptors and chimeric antigen receptors are all showing great promise in the treatment of a variety of malignancies.

Further, the elegant identification and characterization of suppressive cell populations such as Treg cells and myeloid derived suppressor cells allowed for an additional avenue for cancer immunotherapy. Suppression or inhibition of these populations could certainly lead to additional therapeutic benefit for patients with cancer.

The logical conclusion from this truly remarkable Symposium is that the future of cancer treatment will most certainly be replete with treatment programs that include immunotherapeutic approaches. The inherently complementary mechanistic nature of the approaches yields clear opportunities for them to be rationally combined to achieve even greater clinical impact. The collegial and collaborative environment of the Princess Takamatsu Cancer Research Fund Symposium has provided an optimal venue for such valuable efforts to succeed.