

Commentary

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Synopsis of the 6th Walker's Cay Colloquium on Cancer Vaccines and Immunotherapy

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Abstract

The 6th annual Cancer Vaccines and Immunotherapy Colloquium at Walker's Cay was held under the auspices of the Albert B. Sabin Vaccine Institute on March 10–13, 2004. The Colloquium consisted of a select group of 34 scientists representing academia, biotechnology and pharmaceutical industry. The main goal of this gathering was to promote in a peaceful and comfortable environment exchanges between basic and clinical science. The secondary benefit was to inspire novel bench to bedside ventures and at the same time provide feed back about promising and/or disappointing clinical results that could help re-frame some scientific question or guide the design of future trials. Several topics were covered that included tumor antigen discovery and validation, platforms for vaccine development, tolerance, immune suppression and tumor escape mechanisms, adoptive T cell therapy and dendritic cell-based therapies, clinical trials and assessment of response. Here we report salient points raised by speakers or by the audience during animated discussion that followed each individual presentation.

The Colloquium as a unique venue to promote cultural exchanges

Walker's Cay, is a minute tropical island in the northernmost part of the Bahamas archipelago of a size barely sufficient for a runway, a lodge and a harbor. Relaxation and introspection are the only option. Visitors make the majority of the population possibly giving Walker's Cay the highest density of scientists per capita in the World on the days of March 10–13, 2004. In that solitude, the Robin Crusoe in each one of us was more than delighted to educate impromptu Fridays on philosophical matters such as signal transduction and growth regulation or more practical ones such as clinical trial conduct and regulatory hurdles. Although apparently indulgent, this atmosphere promoted comradery and intense exchange of ideas based on the simple principle that scientists, like

most human beings, cannot refrain from talking. Formal presentations served topics for discussion during subsequent free intervals when participants could cluster or disperse at their leisure to deepen their discussions. The key element of this successful recipe was, rather than the confinement, the selection of participants from distant disciplines so that most had never met each other before nor known each other's work. When the time came to leave Walker's Cay, most of us had gained new friends, a better understanding of advances carried by our peers and, more practically, new collaborations.

The Keynote lecture

Rolf Kiessling (Karolinska Institute, Stockholm, Sweden) opened the Colloquium delivering the keynote lecture about: "*The Her-2/neu gene as a target for tumor vaccina-*

tion". Her-2/neu (HER-2) is a 185 kD receptor-like glycoprotein that is over-expressed by a proportion of tumors such as breast, ovarian, gastric and colorectal carcinomas with a frequency of approximately 20 to 30%. Its over-expression is associated with malignant transformation of epithelial cells and the frequency of over-expression varies among types of cancer, but universally represents a marker of poor prognosis. The critical role of HER-2 in epithelial oncogenesis and its selective over-expression on malignant tissues makes it an ideal target for immunotherapy. Antibodies and T cells reactive to HER-2 are known to naturally occur in patients with HER-2 positive tumors, confirming the immunogenicity of the molecule. Both antibodies as well as T cells reactive to HER-2 have been utilized for immunotherapy of HER-2 positive tumors. The "humanized" monoclonal antibody Herceptin has been tested in several clinical trials and found to be an effective adjuvant therapy for HER-2 positive breast and ovarian cancer patients. However, the frequency of patients responding to Herceptin is limited and a majority of patients initially responding to Herceptin develop resistance within a year of treatment. The use of vaccination strategies that generate T cell responses with or without accompanying antibody responses may serve to mitigate the problem. Various strategies for generating T cell-mediated responses against HER-2 are currently being examined in animal models [1] or in clinical trials [2]. Clinical trials based on Her-2/neu vaccination are ongoing at the Karolinska Institute and include a pDNA vaccination with full length Her-1 combined with GM-CSF and IL-2 in patients with advanced breast cancer also on Herceptin treatment and a multi-CTL epitope peptide vaccination combined with GM-CSF and IL-2 in patients with advanced ovarian cancer. Besides these clinical studies strategies are being explored experimentally to avoid epitope loss tumor variant using a multiple epitope approach covering more than one oncogene, increasing the binding affinity of known epitopes through HLA anchor motif substitutions [3] and testing new adjuvants that may include CpG motifs, construction of "smart" viral vectors containing co-stimulatory molecules or cytokines and the implementation of prime-boost strategies using combinations of plasmid DNA, viral vectors or protein products. A new strategy recently explored in the use of polyoma VP1/VP2Her-2 viral like particles that have shown effectiveness in animal models. Finally, strategies should be implemented to avoid tumor escape. This may be quite complex and involve the understanding of tumor as well as host physiology. Several dogmas should be re-assessed such as, for instance the relevance of MHC expression down-regulation and its relationship with that of pro-inflammatory cytokines such as IFN- γ in the tumor micro-environment [4]. At the same time tumor induced immune dysfunction should be taken into account and,

therefore, vaccination strategies should be combined with treatment capable to reverse immune suppression.

Target antigen discovery and validation (Chair: Martin Kast)

Robert Vonderheide (Abramson Family Center Research Institute, University of Pennsylvania, Philadelphia, PA) talked about his work on: "*Telomerase as a target for cancer immunotherapy*". High-level expression of the telomerase reverse transcriptase (hTERT) are observable in >85% of human cancers, in contrast to its restricted expression in normal adult tissues. This observation points to hTERT as a broadly applicable target for cancer immunotherapy. Cytotoxic T lymphocytes (CTL) recognize peptides derived from hTERT and kill hTERT+ tumor cells of multiple histologies in vitro. Moreover, because survival of hTERT+ tumor cells requires functionally active telomerase, hTERT may serve as a prototypic immune target for which mutation or loss as a means of escape may be incompatible with sustained tumor growth. Results of two phase I studies of hTERT vaccination in cancer patients were reported. In the first study (now closed), seven HLA-A2 patients with advanced cancer (5 with prostate cancer, 2 with breast cancer) were vaccinated subcutaneously with autologous monocyte-derived dendritic cells loaded ex vivo with the hTERT HLA-A2 binding peptide I540. As measured by peptide/MHC tetramer, ELISPOT, and cytotoxicity assays, hTERT specific T lymphocytes were induced in 4 of 7 patients. Tetramer-guided high-speed sorting and polyclonal expansion after vaccination achieved highly enriched populations of hTERT-specific cells that killed tumor cells in an MHC-restricted fashion. Despite concerns of telomerase activity in rare normal cells, no significant toxicity was observed, including in the bone marrow. Partial tumor regression in one patient with breast cancer was associated with the induction of CD8+ tumor infiltrating lymphocytes. In a second study (ongoing), patients with advanced breast cancer were vaccinated subcutaneously with I540 peptide emulsified in adjuvant with GM-CSF. Among 9 patients treated thus far at the first or second dose level, no serious adverse events have been observed. Injection site reactions and tumor pain following I540 peptide vaccination have occurred (in contrast to our first trial). In one patient, tumor infiltrating lymphocytes associated with marked tumor necrosis have been observed after vaccination. The lymphocytic infiltrate included CD8+ and CD4+ T cells, macrophages, dendritic cells, plasma cells, and B cells. Based on flow cytometric tetramer analyses, 7%–10% of infiltrating CD8+ T cells were specific for hTERT I540 peptide after vaccination and were persistent for 7+ months. These results demonstrate the immunological feasibility of targeting telomerase as a tumor rejection antigen and provide rationale for telomerase vaccine trials in healthier cancer patients [5].

Robert Bright (Texas Tech University, Lubbock, TX) discussed: "*Prostate cancer antigen discovery*". In particular, he presented a novel strategy for the identification of cancer-specific proteins that could serve as molecular target of vaccination. The significance of his model is based on the ability to grow and characterize cancer cells from primary tumors which notoriously are difficult to expand *in vitro*. Dr. Bright presented an ingenious method for the immortalization of cancer cell lines through the transduction of viral oncogenes such as the E6 and E7 sequences of Human Papilloma Virus 16 based on a retroviral vector [6]. In this fashion, several tumor and normal prostate lines could be developed that could then be used to identify cancer-specific proteins using subtractive hybridization and differential message display. Several new prostate TAA, to include TPD52 (human and murine) and NY-CO-25 were identified as either over expressed or uniquely expressed in tumor cell lines. Tumor expression of candidate TAA was routinely confirmed by RT-PCR compared to normal cell lines and commercially available normal tissue panels, as well as with immune fluorescence and western analysis when specific antibodies were available. Immunogenicity and therapeutic potential; of novel prostate TAA TPD52, was assessed in mice immunized with DNA encoding the murine homologue. In separate experiments, immunized animals failed to reject syngeneic 4T1 mammary tumor cells unless the tumor cells simultaneously expressed a second immunogenic tumor antigen, suggesting in this case that mD52 DNA vaccination induced some degree of anti-tumor immunity which led to an immune response to the second tumor antigen (not included in the vaccine). Relative immunogenicity of the novel prostate TAA NY-CO-25 was tested using *in vitro* sensitization technology and identified a single HLA-A2 restricted P9-modified (T to V) synthetic peptide capable of inducing CTL with tumor reactivity. The systematic development of methods for the successful establishment of tumor and normal cell lines from primary and metastatic cancer may help the identification of novel tumor antigens particularly in those cases in which availability of tumor cell lines is the limiting factor [7].

Maurizio Chiriva-Internati (Texas Tech University, Lubbock, TX) presented his experience in: "*The complex validation of discovered cancer testis antigens*". The salient point of his presentation was that although cancer testis antigens are believed to be specifically expressed only by tumors and be germinal cells in the testis, comprehensive analysis of tissue specimens from most organs identified a protein cross-reacting to a cancer-testing antigen-specific antibody. Sperm protein 17 (Sp17) could be an ideal target for the treatment of multiple myeloma and ovarian cancer, because it seems to have a very restricted distribution in healthy tissues and *in vivo* clinical safety could be deduced from the apparent lack of any pathological con-

dition in men who develop anti-Sp17 antibodies after undergoing vasectomy. The expression of cancer associated antigens has mainly been studied at the level of gene expression. However, this molecular approach does not allow the quantification of cancer cells that are positive for CT antigens. On the contrary, the availability of specific antibodies enables the recognition of the antigen within examined tissues, highlighting not only the quantity but also the type of cells expressing that antigen. Sp17 is strongly expressed in germinal cells and in the flagella of spermatozoa. Because a common origin of cilia and flagella in eukaryotes has been proposed, the expression of Sp17 was investigated in human ciliated epithelia. Indeed; Sp17 was detectable in ciliated epithelia of the respiratory airways and both the male and female reproductive systems questioning the usefulness of Sp17 in immunotherapy protocols. Therefore, it was proposed that validation of possible immunological targets for clinical use should depend on

- *Discriminating* the cell types expressing the antigen on the basis of the morphological visualization of all of the parts making up the *organ* under investigation;
- *Discriminating* the antigen's sub-cellular localization (nucleus, cytoplasm and plasma lemma);
- *Mapping* antigen expression in all of the *organs* making up the living *organism*;
- *Estimating* the percentage of normal cells and their neoplastic counterparts expressing the antigen;
- *Evaluating* the dynamics of antigen expression at the level of the *cell cycle*, the *physiological status of the organism* and the *process of aging*.

Platforms of vaccine development (Chair: Matt Mescher)

Matt Mescher (University of Minnesota, Minneapolis, MN) presented his view on: "*Tumor immunotherapy with large multivalent immunogen: murine models and clinical trials*". In particular, he presented an interesting *in vivo* mouse model demonstrating that 3 signals are necessary for T cell activation. Effective activation of CD8 T cells by sub-cellular antigen or purified class I MHC protein/peptide complexes requires that the Ag be presented on a surface of cell-size dimensions. Administration of plasma membrane tumor Ag immobilized on five-micron microspheres (Large Multivalent Immunogen; LMI) substantially increased generation of CTL responses to syngeneic murine tumors, resulting in decreased tumor growth and extended survival. A phase I trial of LMI treatment of melanoma, using *in vitro* grown melanoma lines as the source of plasma membrane antigen, showed LMI

to be nontoxic and have some immunological activity. Increased CTL precursor frequency was found for eight of fifteen patients [8]. A trial is currently underway examining treatment of melanoma and renal carcinoma using autologous tumor as the source of membrane Ag. Ongoing studies of murine tumor models, using adoptive transfer of TCR transgenic cells to visualize CD8 T cell responses in vivo, demonstrated that LMI prepared using defined class I/peptide complexes are effective for treatment of tumor-bearing mice and allow to define strategies for improving the efficacy of LMI therapy, including co-administration of cytokines and incorporation of co-stimulatory ligands onto the microspheres. Insights gained from studies visualizing vivo CTL responses in different tumor models are suggesting that effective strategies for immunotherapy will depend upon the status of the patients tumor specific CD8 T cells at the time of treatment, with different approaches being indicated depending upon whether the cells are naïve, Ag-experienced effector cells, or cells that have interacted with Ag and become tolerant. His work provides fundamental information for the interpretation of the clinical finding that in most cases vaccination-induced T cells do not seem to be capable to eradicate cancer possibly because they do not achieve a sufficient status of activation at the tumor site [9].

Tibor Keler (Medarex, Inc., Bloomsbury, NJ) talked about the: "Use of antibodies to target antigens for efficient cross-priming of anti-tumor T cell responses". The proper uptake, processing and presentation of TAA by antigen presenting cells (APC) are critical for induction of potent CTL and helper T cell responses. In particular, eliciting CTL responses to exogenous protein antigens has been challenging. Human antibodies were developed to the mannose receptor and to the Type I Fc receptor (CD64) as a means to deliver antigens for enhanced uptake, processing and presentation of antigens by dendritic cells. Targeting antigens to these endocytic receptors on DC results in efficient presentation of the TAA in context of MHC class I and class II molecules. In particular, co-cultures of autologous DC and T cells from healthy volunteers and cancer patients can elicit CTLs that recognize and lyse tumor cells expressing the TAA and sharing at least one HLA molecule. These recombinant vaccines offer a novel approach for eliciting cellular and humoral responses to defined protein antigens [10].

W Martin Kast (University of Southern California, Los Angeles, CA) presented data supporting the: "Anti-tumor efficacy of Venezuelan equine encephalitis virus replicon particles encoding mutated HPV16 E6 and E7 genes". An effective vaccine for treating human papillomavirus (HPV)-associated malignancies such as cervical cancer should elicit strong T cell-mediated immunity (CMI)

against the E6 and/or E7 proteins necessary for the malignant state. A vaccine was developed based on Venezuelan equine encephalitis (VEE) virus replicon particle (VRP) encoding the HPV16 E6 and E7 genes. VLP immunogenicity and anti-tumor efficacy was tested. The E6 and E7 genes were fused to create one open reading frame and mutated at four or at five amino acid positions to inactivate their oncogenic potential. VRP encoding mutant or wild type E6 and E7 proteins elicited comparable cytotoxic T lymphocyte (CTL) responses to an immunodominant E7(49–57) epitope and generated comparable anti-tumor responses in several HPV16 E6(+)E7(+) tumor challenge models: protection from either C3 or TC-1 tumor challenge was observed in 100% of VRP-vaccinated mice. Eradication of C3 tumors was observed in approximately 90% of mice following therapeutic VRP vaccination. Eradication of HLF16 tumors lacking the E7 (49–57) epitope was observed in 90% of human leukocyte antigen (HLA)-A*0201 transgenic mice following vaccination. Finally, the predicted inactivation of E6 and E7 oncogenic potential was confirmed by demonstrating normal levels of both p53 and retinoblastoma proteins in human mammary epithelial cells (MEC) infected with VRP expressing mutant E6 and E7 genes. These promising results support the continued development of mutant E6 and E7 VRP as safe and effective candidates for clinical evaluation against HPV-associated disease [11].

Alain Luxembourg (Ichor Medical Systems, San Diego, CA) described a novel method for the: "Potentiation of DNA vaccines and cancer immunotherapy by electroporation". DNA vaccines are a promising method for vaccination against cancer antigens. They are appropriate to elicit T cell responses, which offer new opportunities for achieving anti-tumor responses. They can comprise multiple antigens, thereby minimizing risk of tumor escape by mutation. The procedure has the potential to enable long-term expression of the antigen, which could help sustain immunological memory. In contrast to cell-based vaccines, DNA vaccination is not patient specific, which facilitates product development, standardization, manufacturing and distribution. However, the translation of these promising findings into clinical products has proven difficult. In particular non viral delivery systems seem to induce insufficient levels of gene expression especially in larger animals and humans. Electroporation (EP), is a potent non-viral delivery technique utilizing the *in vivo* application of electrical fields to enhance intracellular uptake of nucleic acids. Based on the dramatic enhancement of DNA delivery provided by EP, this technique may provide a means to overcome some of the traditional hurdles associated with the use of plasmid DNA vaccines. In order to be considered a viable platform for DNA-based immunization in the clinical setting, a method for effective, reproducible EP-mediated DNA vaccine delivery

must be provided. In addition, the system must be convenient, well tolerated, and cost effective. Ichor's proprietary TriGrid™ intramuscular delivery technology addresses these issues by providing an integrated, automated application system. The system consists of three components: a Pulse Stimulator, an Integrated Applicator, and a single use Application Cartridge. Of principal importance, Ichor's technology provides a user independent means of application, assuring effective and reliable propagation of the EP-inducing electrical fields at the site of agent administration.

Sally Mossman (Corixa Corporation, Seattle, WA) reported on Corixa experience in: "*WT-1 cancer vaccine development: protein-based approaches to inducing comprehensive immunity*". Gene based vaccines, such as viral vectors, have proved highly efficient at generation of CD8⁺ T cell responses, particularly in mouse models, but can be deficient at inducing a potent CD4⁺ helper T cell response. In addition, viral vectors can be used effectively only a limited number of times due to induction of vector-specific neutralizing responses in the host. For these reasons effective antigen delivery may require the administration of recombinant protein antigen into the Class I pathway, while simultaneously inducing strong CD4⁺ T cell responses and high titers of serum antibody. Protein-based approaches afford the opportunity for delivering multiple boosting immunizations. WT-1 is a tumor-associated antigen first identified in Wilm's Tumor. It represents a promising target for development of a therapeutic leukemia vaccine since its expression is highly tumor-specific. A truncated WT-1 construct in which the highly conserved zinc finger region was deleted resulted in dramatically improved immunogenicity *in vivo*. Two different protein-based vaccine approaches were able to induce a WT-1 specific HLA-A2 – restricted CD8⁺ T cell response, high levels of antigen specific IFN- γ secretion from CD4⁺ T cells and significant titers of IgG1 and IgG2a WT-1-specific antibodies in HLA-A2 transgenic mouse models. The first approach utilized a potent adjuvant formulation consisting of the combination of TLR4 and TLR9 agonists in a squalene oil emulsion. The second approach employed WT-1 protein co-encapsulated in a poly(lactide co-glycolide) microsphere with MPL® adjuvant. This latter vaccine formulation in particular is likely to be extremely safe and non-reactogenic in the clinic, and should provide an efficient method for immunizing patients with WT-1-positive hematologic malignancies.

Gerry Rowse (Stressgen Biotechnologies, Victoria, BC, Canada) summarized the use of: "*Hsp-fusion proteins: a platform technology for the treatment of chronic viral diseases and cancer*". There are likely many different reasons for the limited success of immunotherapeutic treatments, but one important problem has been the difficulty of generat-

ing strong, sustained immune responses of the correct immune polarity to key antigenic targets on diseased cells. Evidence suggests that to be effective, an immune response against viral disease and cancer must involve a strong cell-mediated response. A platform technology was developed for the immunotherapeutic treatment of chronic viral diseases and cancer based on the concept of fusing antigenic molecules to highly immunogenic bacterial heat shock proteins (CoVal™ fusions). The fusion of antigens to heat shock proteins (Hsp's) results in the development of Th1-biased immune responses and the generation of cytolytic T lymphocytes even when Hsp-fusion proteins administered without adjuvants. HspBCor is a CoVal™ fusion protein engineered from HBV core antigen (HBc) and Hsp65 from *Mycobacterium bovis* BCG. Re-stimulated splenocytes from C57BL/6 mice immunized with HspBCor in saline displayed a high level of H-2Kb-restricted lytic activity. HspBCor was similarly effective at eliciting HLA-A2-restricted CTL in HLA-A2Kb transgenic mice. HspBCor-primed CTL lysed both peptide-pulsed and HBc-transfected target cells, which express HBc endogenously. IFN- γ was also released by HspBCor-primed CTL. Induction of CTL was further evaluated in HBV transgenic mice, which are commonly employed as a model of chronic HBV infection. A single injection of HspBCor was observed to elicit CTL in a proportion of transgenic mice, despite the immunological tolerance to HBV antigens in these animals. These data support the hypothesis that the CoVal™ fusion protein, HspBCor, will be an effective agent in the immunotherapy of chronic HBV infection and its resultant liver cancer

John Vasilakos (3M Pharmaceuticals, St Paul, MN) reported that: "*Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8*". In particular, John discussed a recent study in which TLR agonists were shown to protect mice against B16 melanoma when used in concert with CD40L. Stimulation of TLR7 and TLR8 appears to modulate the function of innate immunity by promoting the production of TNF- α and interleukin-1 and MIP-1 α (TLR4 and TLR8) or IFN- α or IFN regulated chemokines such as IP-10 and I-TAC (TLR7). Th eTLR7 agonists directly activates plasmacytoid DC and B-cells and, to a lesser extent monocytes. Conversely, TLR8 agonists directly active myeloid DC, monocytes and monocyte-derived DC. *In vivo*, small molecule TLR7 agonists enhance Th1-like immunity and activate class I-restricted CD8 T-cells in mice. CD8 T-cell activation directly correlates with anti-tumor immunity. It also appeared that most of the effect of TLR 4/8 agonists is mediated through activation of the NF- κ B pathway while it remains unclear which is the predominant pathway utilized by TLR-7 agonists. One of the difficulties in interpreting the data regarding the mechanism of action of TLR agonists *in vivo* in animal models is their stringent species specificity and

future studies should consider the study of their mechanism of action directly in humans [12].

Tolerance, immune suppression and tumor escape mechanisms (Chair: Hyam Levitsky)

Tyler Curiel (Tulane University, New Orleans, LA) presented: "Tumor-mediated immune escape and immune suppression". Recent evidence suggests that tumors actively defeat specific immunity through a variety of mechanisms, most of which remain poorly understood. In human ovarian cancer, it was demonstrated that tumor-conditioned dendritic cells (DC) are dysfunctional in many regards, including inducing suppressive IL-10-producing T cells. Tumor plasmacytoid DC are conditioned through tumor stromal-derived factor-1 to induce IL-10-secreting T cells. Tumor myeloid DC are conditioned through tumor VEGF and microenvironmental macrophage IL-10 to express B7-H1, which induces T cell IL-10 and inhibits tumor associated antigen (TAA)-specific immunity. The T cells induced by these tumor-conditioned DC displayed many properties of regulatory T cells. CD4⁺CD25⁺ Tregs are elevated in many humans with cancer, although their functional significance is unclear. Human tumor CD4⁺CD25⁺ Tregs block TAA-specific immunity *in vivo*, and are associated with reduced cancer-specific survival in humans. Endogenous tumor-mediated suppressive functions may explain the relatively poor clinical results in most cancer vaccine trials. Ontak is a fusion protein consisting of the CD25-binding domain of IL-2 fused to the active domain of diphtheria toxin. Ontak kills CD4⁺CD25⁺ Tregs and thus improves immunity in cancer patients. Three patients (one each with advanced-stage cancer of the ovary, breast or lung) received a single intravenous infusion of 9 µg/kg Ontak. Patients had been heavily treated, but had received no cytotoxic drugs, radiation therapy or immune modulating agents for at least 30 days prior to study entry and had no autoimmune disease or active immunologic processes aside from their cancer. Prior to infusion, the mean prevalence of CD4⁺CD25⁺ cells in blood CD4⁺ T cells blood was 9.0 %, which fell to a mean of 5.2 % three days after infusion ($P = 0.0007$). Prior to infusion of Ontak, there were a mean of 27.6 CD4⁺CD25⁺ cells/mm³ in blood which fell to 11.9 CD4⁺CD25⁺ cells/mm³ three days after Ontak. The total number of CD4⁺CD25⁺ cells decreased approximately 50% in each patient. The prevalence of interferon- γ CD8⁺ T cells increased a mean of 40% accompanied by a corresponding increase in the absolute number of interferon- γ CD8⁺ T cells after Ontak. The prevalence of the proliferation marker Ki-67⁺ T cells in blood increased significantly from 0.93% before Ontak to 4.5% of all T cells three days after treatment. The treatment was well tolerated. This strategy, or others designed to defeat tumor-mediated immune suppression, may be combined with active immunization for optimal cancer vaccine efficacy [13].

Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY) presented his novel strategy for: "Monitoring HLA class I antigen-tumor antigen peptide complexes on melanoma cells". To date, a number of studies have clearly demonstrated that HLA class I antigens as well as components of the antigen processing machinery (APM) are frequently down-regulated and/or lost in malignant lesions. The information remains scant because of the need of developing reagents that can be used in paraffin sections representing most available clinical material. Recent work from his laboratory lead to the production of monoclonal antibodies utilizing a semi-synthetic phage display antibody library able to measure the expression of specific HLA class I-tumor antigen peptide complexes (in this particular case a peptide epitope from the melanoma associated antigen MART-1). Preliminary data suggested that there is poor correlation between HLA class I expression on number of HLA-MART-1 complexes suggesting that the simple analysis of HLA complexes may be misleading because it does not take into account the efficiency and individual tumor antigen processing and presentation by cancer cells.

Hyam Levitsky (Sidney Kimmel Comprehensive Cancer Center, John Hopkins School of Medicine, Baltimore, MD) talked about the: "Visualization of tumor antigen specific CD4⁺ T cell suppression". CD4⁺ tumor-specific T cells have impaired function and cannot be primed *in vivo*. Using the adoptive transfer of CD4⁺ T cells from TCR transgenic mice labeled with CFSE it was possible to demonstrate the kinetics of *in vivo* proliferation of T cells in naïve non-tumor bearing animals and tumor bearing mice. Adoptive transfer of transgenic CD4⁺ T cells recognizing an MHC class II restricted epitope of influenza hemagglutinin (HA) into mice harboring a systemic B cell lymphoma expressing HA (A20HA) demonstrated evidence of antigen recognition. Clonotype⁺ T cells underwent a modest expansion, and displayed a phenotype consistent with antigen-experienced T cells. Analysis of cell division by CFSE dilution revealed that only a minority of the HA-specific T cells enter the cell cycle, in spite of a progressively expanding systemic tumor burden. Sorting HA-specific CD4⁺ T cells from A20HA bearing mice into CFSE_{hi} (undivided) and CFSE_{lo} (divided) populations identified that while the former respond to HA peptide equivalently to naïve HA-specific T cells *in vitro* (proliferation and IL-2 production), the divided cells displayed markedly impaired responses. Furthermore, while immunization of A20HA bearing mice with HA-expressing virus increased the number and fraction HA-specific T cells that have divided, this "primed population" also had a markedly impaired proliferative response to peptide *in vitro*, diminished IL-2 production, and failure to make interferon- γ as compared to CFSE_{lo} HA-specific T cells from virus primed, non-tumor bearing mice. Limited gene pro-

filing of CFSE₁₀ HA-specific T cells from A20HA bearing mice demonstrated less mRNA for IL-2, CD40L, t-bet, and IFN- γ following TCR cross-linking than similarly treated naïve or virus primed T cells from non-tumor bearing mice. In contrast, message for IL-10, GITR, foxp-3, and LAG-3 were markedly increased in tumor-specific, antigen-experienced T cells. *In vitro* mixing of CFSE₁₀ HA-specific T cells purified from A20HA bearing mice with naïve HA-specific "responder" T cells resulted in profound suppression of proliferation, and IL-2 production. Remarkably, divided HA-specific T cells from A20HA bearing mice also suppressed IFN- γ production of CD4⁺ effector cells purified from virus primed, non-tumor bearing mice. Trans-well experiments demonstrated the requirement for cell:cell contact for suppression. Suppression was antigen-specific, as mixing CFSE₁₀ HA-specific T cells from A20HA bearing mice with naïve OVA-specific CD4⁺ T cells had no impact on proliferation or IL-2 production when pulsed with both HA and OVA peptides *in vitro*. Finally, sorted CFSE₁₀ HA-specific CD4⁺ T cells (thy1.1^{+/+}) obtained from A20HA bearing mice were mixed with naïve HA-specific responder T cells (thy1.1^{+/1.2+}) and injected into non-tumor bearing recipients, which were then primed with HA-expressing virus. Whereas both populations divided in response to infection, the presence of the putative suppressor population significantly diminished the accumulation of the responder T cells and blocked their differentiation into INF- γ producing Th1 cells.

Stephen Schoenberger (La Jolla Institute, San Diego, CA) presented: "A TRAIL of death for helpless CTLs". The goals of cancer immunotherapy could be significantly advanced through a detailed and mechanistic understanding of the immunobiology of cytotoxic CD8⁺ T lymphocytes (CTL). Whereas the initial clonal expansion of CTL can proceed in the absence of CD4⁺ T lymphocytes, their ability to undergo secondary expansion upon re-exposure to antigen is wholly dependent on the presence of CD4 T help during priming. Thus, CTL primed in the absence of T help ("helpless" CTL) can function as cytotoxic effector functions in a recall response, but do not subsequently undergo secondary expansion. The helpless CTL following re-stimulation undergo an abortive expansion leading to death via a caspase-dependent apoptosis. The relevance of the known pro-apoptotic pathways to the death of helpless CTL was then investigated using a genetic approach. These experiments ruled out a role for either the so-called "passive" (mitochondrial) death pathway the Fas-Fas-L or TNF-R death receptor pathways in the disappearance of helpless CTL following re-stimulation. In contrast, the combined molecular and genetic data indicate that the TRAIL/TNF-R2 (DR5) pathway is responsible for the activation-induced cell death (AICD) observed when helpless CTL encounter antigen for the second time. The results provide new insights into the role of TRAIL in

immune regulation and reveal new targets for therapeutic intervention [14].

Hans Schreiber (University of Chicago, Chicago, IL) presented: "Bystander elimination of antigen loss variants in established tumors". T cells activated in mice bearing tumors expressing high levels of antigen can kill synchronous tumors with low antigen expression. These tumors would not be killed in the absence of concomitant immunity. In humans, it is likely that most tumors are expressing relatively low level of antigens and this is why they can grow. Possible, the enhancement of antigen expression or presentation in some tumors through local stimulation by cytokines or other factors could induce anti-tumor immunity and subsequent epitope spreading activating T cells against several tumor antigens present in the tumor. These may in turn be capable of destroying distant tumors. This hypothesis has not been tested in humans as yet though several groups have advocated increasing the immunogenic potential of individual lesions *in vivo* through local immune stimulation as a possible tool to induce immune responses toward synchronous autologous metastases [15].

Eduardo Sotomayor (Moffitt Cancer Center, Tampa, FL) presented: "Molecular strategies to overcome tumor-induced immune tolerance and enhance the efficacy of cancer vaccines". His work suggests that most tumors are infiltrated with tolerogenic antigen presenting cells with high expression of STAT-3, cKIT and histone de-acetylase. It could be possible to revert this process by administering inhibitors of these molecules in combination with vaccination. Several putative molecules were discussed such as LAQ-842, an inhibitor of histone de-acetylase [16].

Hua Yu (Moffitt Cancer Center, Tampa, FL) presented her work on the: "Role of Stat3 in oncogenesis, tumor immune evasion and immunotherapy". For a tumor to reach clinical stage, it must be able to resist apoptosis, sustain proliferation, attract nutrients through angiogenesis and evade immune detection. As a point of convergence for many oncoproteins, signal transducer and activator of transcription 3 (Stat3) protein participates in regulation of all of these processes and is activated in a very large number of cancers. Stat3 promotes tumor cell growth by regulating genes critical for survival and proliferation. A master regulator of vascular endothelial growth factor, Stat3 is also critical for tumor angiogenesis. Blocking Stat3 leads to tumor cell apoptosis, tumor vessel impairment and tumor regression. While the mechanism linking oncogenesis and immune evasion has been elusive, our recent data indicate that oncogenic signaling pathways use Stat3 to shield cancer from the immune system. Stat3 activity in tumor cells inhibits the production of pro-inflammatory cytokines and chemokines while inducing the release of factors that

inhibit dendritic cell (DC) maturation through activation of Stat3 in DCs. Blocking Stat3 in tumor cells allows production of inflammatory mediators, leading to activation of both innate and adaptive immune responses. Moreover, inhibition of Stat3 in immune cells unleashes potent anti-tumor immune responses. Specifically, *in vivo* deletion of the *Stat3* alleles in bone marrow cells enables DCs to escape tumor-induced maturation inhibition, leading to T cell activation. Additionally, the ability of *Stat3*^{-/-} NK cells and neutrophils from tumor-bearing mice to kill tumor cells is considerably enhanced compared to their *Stat3*^{+/+} counterparts. Growth of B16 and MB49 tumors in mice with *Stat3*-null bone marrow cells is significantly inhibited. In summary the blockage of Stat3 – in either tumor or immune cells – stimulates anti-tumor immune responses. With the emergence of Stat3-specific pharmacologic inhibitors, molecular targeting to modulate host Stat3 activity is expected to evoke anti-tumor immunity and strengthen the efficacies of a variety of immunotherapeutic approaches [17].

Adoptive T cell therapy and dendritic cell (DC) based therapies (Chair: Michael Nishimura)

Bernard Fox (Earle A. Chiles Research Institute, Portland, OR) proposed to: "*Exploit lymphopenia to reboot immune response to cancer*". A primary reason for the failure of cancer vaccine strategies is because the magnitude of the anti-tumor immune response is insufficient to mediate tumor regression. Recently, we described a novel strategy to augment priming of tumor-specific T cells by vaccinating lymphopenic mice that had been reconstituted with 2×10^7 spleen cells. Tumor vaccine-draining lymph nodes (TVDLN) of reconstituted-lymphopenic mice (RLM) vaccinated with D5-G6, a GM-CSF-secreting line of the poorly immunogenic B16BL6-D5 (D5) melanoma, contained an increased number of activated T cells (OX-40, LY6C, CD62L^{low}) [18]. Following *in vitro* activation and expansion these TVDLN exhibited an increased frequency of tumor-specific CD4 and CD8⁺ T cells that correlated with significantly ($p < 0.05$) increased therapeutic efficacy in adoptive transfer studies. This strategy was also effective in an active-specific vaccine strategy, significantly augmenting the generation of a protective immune response against the weakly immunogenic B16-F10 melanoma [19]. While initial studies were performed in congenitally lymphopenic RAG-1 or irradiated mice, current studies examined whether lymphopenia induced by chemotherapy would be effective in our RLM strategy. Lymphopenia induced by cyclophosphamide (CY) was tested for its effect on the number of activated T cells in TVDLN of RLM. One or two days following drug administration mice were reconstituted with spleen cells and vaccinated with D5-G6. TVDLN were harvested 8 days later and T cells analyzed for expression of activation markers. The addition of CY substantially increased the percentage of

CD62L^{low} / CD44⁺ T cells with the greatest increase observed at the highest dose of CY. A similar correlation was observed between CY dose and frequency of tumor-specific T cells with therapeutic efficacy (Ma, J., et al., submitted). While the RLM strategy clearly augments the therapeutic effectiveness of vaccine strategies, these studies reconstituted lymphopenic mice with spleen cells (SC) from naive mice. Using SC from systemic-tumor-bearing mice (TBM) in the RLM strategy resulted in reduced frequencies of tumor-specific IFN- γ secreting effector T cells that could not mediate regression of pulmonary metastases in adoptive transfer studies. The question was then addressed of whether depletion of CD25⁺ cells from SC of TBM used in the RLM strategy would improve the anti-tumor effect. Effector T cells generated from mice reconstituted with CD25-depleted TBM SC had their frequency of tumor-specific IFN- γ secreting T cells and therapeutic efficacy restored (Poehlein, C.H., et al., submitted). Clinical trials of the RLM strategy are presently under preparation, these findings may provide information that can be used to monitor and/or improve the translation of this strategy to the clinic. Clinical trials of this strategy are planned in melanoma, prostate cancer (W.J. Urba and B. Curti, Earle A. Chiles Research Institute), non small cell lung cancer (D. Ruettinger, Klinikum Grosshadern, LMU, Munich, Germany) and ovarian cancer (J. Ma, Xi'an Jiaotong University, Xi'an, China) patients.

Michael Nishimura (University of Chicago, Chicago, IL) presented his work on: "*T cell receptor gene modified T cells for the immunotherapy of cancer*". His work demonstrated that TCR with high affinity for their ligand become CD4 or CD8. For instance, a CD4 clone recognizing a melanoma antigen could interact in an HLA class I restricted fashion with tumor cells. Selection and cloning of T cells characterized by high affinity and independence from co-receptor support may be a useful tool to identify the most relevant anti-cancer agents. Genetic transfer of such constructs into T cell for adoptive transfer would have several advantages. Not only it would provide a high avidity T cell receptor for any patient but knowledge of the specific sequence would allow tracking of the localization of the adoptively transferred T cells using clonotype specific probes for quantitative real-time PCR or other detection methods. Finally, ability of producing T cells expressing two T cell receptors could be considered so that T cells bi-specific for a viral and, at the same time, a tumor antigen could be adoptively transferred allowing one to be activated by the other specificity using various vaccination models [20].

Michel Sadelain (Memorial Sloan-Kettering Cancer Center, New York, NY) presented: "*Targeting tumors with genetically enhanced lymphocytes*". Two scFv/ ζ chain-based chimeric antigen receptors specific for CD19 (B cell line-

age-specific) or PSMA (Prostate epithelium-specific marker) could be introduced in T lymphocytes to eradicate tumors in various mouse models. Although redirection of the T cell response through TCR transfer other requirements appeared necessary including co-stimulation with CD880 and IL-15 which increased the persistence of adoptively transferred cells. The study showed that if T cells are given in sufficient number and properly activated, further co-stimulation *in vivo* is not necessary and they can survive for a period long enough to completely eradicate tumor deposits [21].

David Urdal (Dendreon Corporation, Seattle, WA) reported on: "*Immunotherapy of cancer: progress in the development of Provenge® in prostatic cancer*". Provenge® is a therapeutic vaccine composed of autologous antigen presenting cells cultured *ex vivo* with a recombinant fusion antigen consisting of Prostatic Acid Phosphatase (PAP) linked to granulocyte macrophage colony stimulating factor. The drug is in the final stage of clinical evaluation in men with androgen-independent prostate cancer. Results from a completed phase 3, double-blind placebo controlled, trial suggest that in men with Gleason ≤ 7 tumors, Provenge delays disease progression, delays the onset of disease related pain and results in a survival advantage when compared to patients who had been randomized to placebo [22].

Clinical trials and immune assessment or response (chair Francesco M Marincola)

Neil Berinstein (Aventis Pasteur, Toronto, ON, Canada) presented the: "*Induction of CD8+ T cell responses to melanoma with tumor-antigen expressing canary pox vectors*". The canary pox vector Alvac was used to induce specific CD8+ T cell responses to melanoma cells bearing the gp100 melanoma differentiation antigen. A full-length cDNA for gp100 was engineered into the Alvac vector with modifications to the HLA A*02010-associated CTL epitopes at positions 209 and 280 to increase binding affinity to the HLA molecule. Thirty-six patients were treated in a multi-centre phase I trial which evaluated prime boost approaches with Alvac and the gp100:209-2M and gp100:280-9V peptides, different routes of administration of the vaccines and whether non-specific T helper adjuvant tetanus toxoid could enhance the responses. The vaccine was found to be safe. Elispot and tetramer assays for T cell responses after *in vitro* cell culture demonstrated: 1) heterologous prime boost immunizations with Alvac 2 gp100M and peptides generated measurable increases in gp100 reactive T cells 2) the viral component of the vaccine was required for T cell induction as peptides alone were completely ineffective 3) intra-nodal administration appeared more effective than subcutaneous vaccination 4) patients receiving tetanus toxoid had lower T cell responses than the non-tetanus

toxoid groups 5) most vaccine-induced responses were not long lived as gp100 reactivity returned to baseline by the end of the study. 6) Classical clinical responses were not seen with the vaccine alone but were seen in 2 of 7 patients (only three of whom had measurable disease) treated with high dose Interferon- α post vaccination. These responses were associated with recall of Gp100 specific CD8 T cells with enhanced functional (lytic) activity [23].

Ivan Borrello (John Hopkins University, Baltimore, MD) showed data demonstrating that: "*Activated marrow infiltrating lymphocytes impart a strong anti-tumor effect on plasma cells and their clonogenic precursors: a novel approach to adoptive immunotherapy*". In multiple myeloma, marrow infiltrating lymphocytes (MILs) can be easily obtained and expanded with significant tumor specificity utilizing an artificial antigen presenting cell consisting of anti-CD3/antiCD-28 antibodies coupled to a magnetic bead. This *in vitro* activation of MILs results in an 8–10 fold increase in tumor specificity as compared to unactivated MILs and contrasts with non-measurable tumor specificity in either activated or unactivated circulating lymphocytes. The anti-tumor activity of activated MILs targeted the terminally differentiated CD138+ plasma cells as well as the myeloma precursor and inhibited growth of myeloma precursors in a clonogenic assay. Activation within the marrow microenvironment is critical to enhancing tumor specificity as isolation of MILs from the marrow and subsequent activation significantly reduced their tumor specificity thereby underscoring the critical requirement of antigen exposure during both the activation and expansion phases. The ability to augment the therapeutic anti-tumor efficacy of adoptive immunotherapy will hopefully translate into clinically measurable advances in the treatment of multiple myeloma and as well as other hematologic malignancies (Anti-myeloma activity of activated marrow infiltrating lymphocytes: a novel approach to adoptive immunotherapy. Noonan, K et al submitted).

Lothar Finke (EMD Pharmaceuticals inc., Durham, NC) reported on: "*An international, randomized phase III clinical trials of STn-KLH (Theraptope®) therapeutic cancer vaccine in metastatic breast cancer patients*". Theraptope is an investigational therapeutic cancer vaccine consisting of a synthetic form of the tumor associated antigen Sialyl Tn (STn) conjugated to the carrier protein keyhole limpet hemocyanin (KLH). In a phase III clinical trial, patients with metastatic breast cancer patients (MBC pts) who had no evidence of disease or non-progressive disease following any first-line chemotherapy were randomized 1:1 to receive adjuvant plus Theraptope or control [adjuvant plus KLH]. All pts received a single IV infusion of cyclophosphamide before vaccine. Primary endpoints were time to disease progression (TTP) and overall survival (OS). Pts were stratified by

disease status and concomitant hormone therapy (HT). The randomization included 1,028 patients: 32% received concurrent HT. Median TTP and OS for Theratope (T) and control (C) pts were slightly but not significantly better in the population of patients receiving HT. Theratope was generally well tolerated. Injection site ulcerations occurred with similar frequency in the Theratope and control groups (17.5% vs. 17.6%); 8% of pts discontinued due to adverse events. A phase II study in ER positive MBC pts receiving HT and Theratope is currently underway.

Kristen Hege (Cell Genesys Inc., South San Francisco, CA) presented: "GVAX cancer vaccines – clinical development". GVAX cancer vaccines are composed of whole tumor cells genetically modified to secrete GM-CSF which has the advantage of a prolonged release over injection of the cytokine at the time of vaccination. Sources of tumor cells for vaccine manufacturing include both autologous cells derived from individual patients as well as allogeneic tumor cell lines. Clinical development of the various GVAX vaccine platforms in prostate, lung, and hematologic cancers will be reviewed. Autologous vaccines have been performed in the context of renal cell cancer, melanoma, prostate and non-small-cell lung cancer. Allogeneic vaccines in the context of prostate and pancreatic cancer. Bystander GVAX refers to the utilization of autologous tumor cells mixed with allogeneic cell lines modified to secrete GVAX and have been utilized in the context of multiple myeloma and AML. In prostate cancer allogeneic vaccines have been shown to induce increases in GM-CSF levels and induce tumor reacting antibodies by Western Blot. In addition, decreases in PSA levels have been noted with an association with a trend to improved survival. Autologous vaccines in lung cancer have been associated with complete responses particularly in the case of alveolar carcinoma and a significant increase in survival [24,25]

Kim Lyerly (Duke University, Durham, NC) discussed: "Antigen specific cellular immune responses following DC-base vaccination". DC were matured with a combination of LPS, CD40L and IFN- γ and various immunizations were performed particularly using a pox-vector encoding CEA or mRNA. Immune monitoring demonstrated a close correlation among assays performed which included ELISPOT, intra-cellular cytokine staining and tetrameric HLA/complexes analysis.

Francesco M Marincola (Clinical Center, National Institutes of Health, Bethesda, MD) described strategies for the: "Monitoring of anti-cancer immune responses". In particular, the NIH experience using tumor antigens recognized by T cells for active immunization trials was discussed. These vaccines mostly left the clinicians and researchers perplexed by the paradoxical observation of the immunization-induced T cells can recognize tumor cells in stand-

ard assays but most often cannot induce tumor regression. Indeed, successful immunization is one of several steps required for tumor clearance but more work needs to be done to understand how T cells can localize and be effective at the receiving end within a tumor microenvironment in most cases not conducive to the execution of their effector function. In fact, metastatic melanoma stands out among human cancers because of its immune responsiveness. Yet, the reason(s) remain(s) unclear. A promising strategy for the understanding of melanoma immune responsiveness could consist of the study of tumor/host interactions *ex vivo* through genetic profiling of serial fine needle aspirate biopsies that allow direct correlation between experimental results and clinical outcome. By prospectively studying the transcriptional profile of melanoma metastases during immunotherapy it was possible to observe that immune responsiveness is pre-determined by an immune reactive micro-environment. Interestingly, the addition of systemic interleukin-2 therapy to active specific immunization seems to increase the frequency of immune rejections of cancer. Functional profiling of the effect of interleukin-2 in tumors suggested that this cytokine induces or enhances the effector function of immunization-induced T cells by causing an acute inflammatory process at the tumor site that can in turn recruit and activate T cells [26]. Future clinical studies should incorporate tools that allow global monitoring of immune responses with high-throughput systems that could take into account the genetic make up of patients, the transcriptional profile of tumor and circulating lymphocytes and the expression of relevant protein products [27-29]

Conclusions

It may appear difficult to make final conclusions given the diversity of the topics covered. However, one remark cannot be passed up; balanced meetings such as the Walker's Cay symposium address the need for intense interaction among basic scientist, clinical researchers and product development favoring in its essence the recognized need for translational efforts for health care improvement [30,31] Several issues could be addressed in the bench to bedside and bedside to bench direction among scientists beyond the specifics of each individual's research so that, when the plane arrived to take us back to the mainland each one of us had a broader vision than when we arrived.

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