**Immunotherapy for the new century**

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**Introductory note**

“Immunotherapy for the new century” was the continuation of a series of international workshops organized by the Center of Molecular Immunology (CIM), in Havana. The previous meetings were in 1994, 1996, 1998, 2000 and 2002 with the participation of scientists from many different countries. The participation in the meeting is generally based on invitations to selected scientists. This edition, IT-2004, was held in the framework of the 10th Anniversary of this center.

IT-2004 was focused on building a vision of cancer immunotherapy for the next ten years. A central topic in the meeting was related to the impact of our current understanding on the complexity of the Immune System (IS) in the development of new cancer immunotherapeutic agents, and how to translate concepts from systems biology to the reductionistic, but operational, approaches of molecular and structural biology. IT-2004 called for an intellectual effort to discover practical consequences from the emerging paradigms concerning IS regulation, autoregulation, and tumor immunology.

During the nineties, two major concepts revolutionized our knowledge on the regulation of the IS: 1) the instructive role of the Innate Immune System (IIS) on the Adaptive Immune System (AIS), and 2) the existence of a peripheral dominant tolerance mediated by regulatory cells. In parallel, new concepts also emerged from tumor immunology: 1) the enlargement of the tumor immuno-surveillance concept to include the IIS, 2) tumor-induced immunosuppression, and 3) tumor-immunoediting.

Thus far, immunotherapy has been shown to increase survival in advanced cancer patients, by controlling disease progression and maintaining a substantial quality of life. An appealing question is to what extent can immunotherapy contribute to control disease progression in different stages of malignant diseases. Possibly, different strategies should be followed to prevent cancer progression, from high risk populations to patients with metastatic disease.

Hence studies on IS regulation, tumor immunology and tumor vaccines were some of the main research areas that were widely addressed during the meeting. These subjects were not only programmed in the oral and poster sessions but they were also brought up in most of the discussions, alluding to the regulatory role of cells from both innate and adaptive IS, the mechanism of tumor evasion and the experience accumulated thus far in tumor immunotherapy.

**Regulation of the innate immune system**

The session devoted to immunoregulation, had two branches: innate and adaptive IS. The IIS is an ancient mechanism of host defense found in essentially every multicellular organism, from plants to humans. In invertebrates, it is the only mechanism of defense. Vertebrates also developed an adaptive immune response, however, the IIS is essential for instructing the cells of the adaptive system (T and B cells) by presenting the antigen in the context of appropriate costimulatory molecules. The IIS developed to not only discriminate self from non-self but more importantly, to discriminate infectious non-self from innocuous non-self. Dendritic cells (DC), and other cells of the IIS, sense and respond to microbial products via the Toll-like receptor (TLR) family. TLRs are an evolutionarily conserved family of cell surface molecules that participate in the innate immune recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are generally unique, chemically diverse products with conserved motifs that are produced by microorganisms. In this context we had, at IT-2004, two very important conferences related to viral immunology and its relationship with the IIS. The first one given by Dr. Sandra Diebold (Cancer Research UK, UK) demonstrated that ssRNA (mimicking viral infection) but also mammalian mRNA are capable of inducing type I IFN via TLR7. However the differential expression of this receptor on plasmacytoid (PDC) and CD8+ DC indicate that this receptor may differ from other pattern recognition receptors in detecting the abnormal localization of ligands rather than structures or motifs absent in the host. In the second talk, Dr. Maria Montoya (EJIVR, UK) analyzed the changes occurring in splenic DC subsets in response to LCMV infection in vivo. She showed that after infection, conventional DC subsets are rapidly activated but trend to undergo apoptosis. Conversely, the number and type 1 interferon production by plasmacytoid DCs increased.

The following conferences were related to the development of adjuvants for cancer immunotherapy based on bacteria and IIS interaction. In this sense Circe Mesa (CIM, Cuba) exposed recent results with an adjuvant called VSSP (Very Small Size Proteoliposome), which combine gangliosides and external membrane vesicles from Neisseria meningitidis in its structure. She showed that these particles induced CTL responses to co-administered peptides and proteins as well as strong antitumor protection when used as an adjuvant of syngeneic tumor cell vaccines. Another approach related to adjuvants was addressed by Prof. Wolf Hervé Fridman (CRB des Cordeliers, France). In this case he described a vector made with the non-toxic B chain of Shigella dysenteriae (STxB) which penetrates into DC and B cells through the Gb3 receptor. In his talk, Prof Fridman stated that STxB is a suitable vector for CTL induction but more importantly he showed tumor...
regression in mice treated with Shiga toxin fused to a tumor-associated antigen, enabling its use in human cancer immunotherapy.

The closing conference was devoted to the goal standard adjuvant CpG and was precisely on the contacts between viral (type I IFNs) and microbial infection as well as innate immunity and adjuvants development. In this talk, Dr. Gunther Hartmann (Ludwigs-Maximilians-University of Munich Ziemsenstrasse, Germany) reviewed that the TLR9 receptor (restrictedly expressed on PDC and B cells) has been established by the IS to detect CpG within certain sequence motifs. Based on the activation of PDC and B cells, they identified three distinct classes of CpG: CpG-A induces type I IFN in PDC, CpG-B is a potent stimulator of B cells and CpG-C combines both properties. With this approach they showed distinct functional profiles of CTL activation. While CpG-B enhanced the frequency of both naive and memory CD8+ T cells, CpG-A only expanded memory CTLs. In the second part of his talk, Dr. Hartmann showed that peritumoral CpG DNA elicited a coordinated response of CD8 T cells and innate effectors to cure established tumors in a syngeneic murine model and that the combination of DC and CpG -based immunotherapy was potent enough to cure even large murine tumors.

**Regulation of the adaptive immune system**

The adaptive branch of the IS composed by cells with variable region receptors (i.e.: T and B lymphocytes) guarantee antigen specificity and memory immunoreactivity. Early studies showed that its own components such as antibodies (Ab) and T cells with different phenotypes were associated with a regulatory function. The potential influence of these mechanisms on tumor immunology and immunotherapy has been extensively discussed in previous editions of this meeting. From the last version of this workshop, experimental and clinical evidence has emerged supporting the relevance of this aspect for tumor biology. Furthermore, our knowledge on regulatory functions has expanded. In spite of this effort, our understanding on the role of dominant tolerance and regulatory cells has not yet led to its direct translation into the clinical setting.

Consequently, it was discussed in a session on the immunoregulation of the adaptive branch of the IS based on the emerging knowledge on dominant tolerance and regulatory cells. Topics addressed in this session were focused on the activation and function of regulatory T and B cells, distinct markers for regulatory cells, dynamics of lymphocyte homeostasis during chemotherapy, immuno-suppressive treatments and other immunodeficient states (e.g.: AIDS), immunoregulatory networks of T and/or B cells and idiotypes connecting the adaptive and innate branches of the IS, the potential contribution of tumors to the strengthening of regulatory mechanisms and the analysis of cancer vaccine strategies based on the immunomodulation of regulatory mechanisms.

Since the Network Theory postulated by Niels K. Jerne, Ab were envisaged as the main feedback mechanism of the IS. Therapeutically, the use of pooled intravenous human IgG (ivIg) in autoimmune diseases has been conceptually associated with this premise. Hans U. Lutz (Swiss Federal Institute of Technology, Switzerland) based on a series of systematic experiments documented that anti-C3 and framework-specific anti-idiotypic naturally occurring antibodies (NAb) contained in ivIg modulated complement amplification. The data suggests that NAb complexes containing 2 anti-C3 and 3 anti-idiotypic NAb inhibits the assembly of C3 convertase presumably by sterically interfering with factor B binding. Thus, this pair of NAb is able to modulate C3 convertase activity. This finding supports the anti-inflammatory effects of ivIg attenuating complement amplification. Furthermore, this finding documented a regulatory mechanism of the adaptive and innate branches of the IS connected through idiotypes.

Experimental data on idiotype networks in a ganglioside-associated model is very limited. However, Rolando Pérez (CIM, Cuba) presented a comprehensive overview on the essential data obtained by targeting cancer in animal models and in patients through N-glycolylated gangliosides using anti-idiotypic mAb. After obtaining a highly immunogenic anti-N-glycolylated ganglioside mAb in syngeneic mice that is able to activate autologous anti-idiotypic T cells, a germ-line encoded Ab2 mAb was produced. This mAb could not induce an antigen-specific Ab response in syngeneic mice where N-glycolylated gangliosides are self-antigens. They may therefore be found a regulatory idiotype that may contribute to natural tolerance to self-glycolipids in mice. Moreover, common amino acid residues are simultaneously involved in antigen recognition, anti-idiotypic interactions, stimulation of T cell proliferation and immunogenicity.

Instead of regulating the immunoresponse by Ab, Amit Bar-Or (Montreal Neurological Institute, Canada) presented a novel regulatory network of B cell effector cytokines in autoimmune diseases. Based on studies in Multiple Sclerosis patients, it was found that B cells appropriately stimulated by sequential B cell receptors and CD40 stimulation proliferate and secrete tumor necrosis factor-α (TNFα) and lymphotoxin (LT) which contribute to a germinal center reaction and thereby amplify the ongoing immune response. In contrast, CD40 stimulation alone -a mimic of a B cell receiving bystander T cell help in the absence of a specific antigen recognition- induces negligible pro-inflammatory cytokines but a significant production of IL-10 that serves to suppress inappropriate immune responses. This result supports an original active immunoregulatory role for B cells that can be targeted therapeutically.

T cell regulation has gained momentum in immunological research. Gérard J. Prud’homme (University of Toronto, Canada) contributed to this field. He was able to induce regulatory T cells (Treg) in autoimmune diabetes-prone NOD mice through DNA vaccination with a plasmid vector encoding an insulin-GAD65 fusion protein (Ins-GAD). This effect was enhanced by co-injecting a second plasmid encoding a mutant B7 molecule (B7-Iwa), which binds to the immunoinhibitory T-cell molecule CTLA-4, but has lost the ability to bind to the costimulatory molecule CD28.
This is consistent with the role for CTLA-4 engaging in generating Treg activity, and represents a promising approach to autoimmune disease prevention. Alternatively, a murine cancer model showed that both the B7.1/Ig and B7.1wa/Ig plasmids, when co-administered with a CEA-plasmid, enhanced tumor rejection and the in vitro anti-CEA response. These results suggest that blocking CTLA-4/B7.1 binding in T cell/T cell interactions blocks negative regulatory signals. Hence, ligating CTLA-4 with either membrane-bound or soluble ligands has a profound effect on the course of DNA vaccination, and can be used to ameliorate disease. The therapeutic blockade of negative signaling (particularly of CTLA-4) increases immunity against tumor antigens constituting a way of altering immune tolerance therapeutically.

Autoimmunity as tumor immunity is under intensive evaluation. Self-Epidermal Growth Factor (EGF) conjugated to a carrier protein (P64k) from Neisseria meningitidis is being tested as a cancer vaccine to induce neutralizing auto-Ab and inhibit EGF-dependent tumor growth. Thus, enhancing the immunoresponse to EGF can in turn contribute to vaccine efficacy that can be achieved on exploring the emerging notions of the contribution of Treg and homeostatic proliferation to immunoregulation. In this line, Enrique Montero (CIM, Cuba) evaluated whether the combination of EGF-based cancer vaccine and chemotherapy could affect immunoregulation. In BALB/c mice it was found that high doses of Adriamycin/Cyclophosphamide administration, which preferentially induce transient CD4+ T and B-cells depletion, reduce the EGF response to the vaccine when given before a primary immunization, as expected. But, surprisingly this response was enhanced 2.5 times when given before a second immunization, at the time when lymphocytes start homeostatic regeneration. Additionally, transient depletion of CD4+CD25+ Treg with an anti-CD25 mAb under the same scheme enhances the Ab response to EGF. Interestingly, the immunoresponse to P64k was not modified by these combinations. These results support the notion that peripheral tolerance is maintained by redundant mechanisms that can be targeted for therapeutic interventions in self-antigens based cancer vaccines.

Awen Gallimore (Cardiff University, UK) addressed the point whether modulation of tumor growth in vivo can be achieved by the innate and adaptive IS. She found that MB16 melanoma cells transfected with FasL can stimulate a rapid inflammatory innate immune response, which leads to effective tumor rejection. Furthermore, this effectively primes the adaptive immune response, resulting in immunity against challenge with the parent cell line. In contrast, inducing inflammation with a mixture of live and dead MB16 cells results in enhanced tumorigenesis and no protection against MB16 challenge. Thus, inflammatory cells can either promote or reject tumor growth depending on the conditions of in vivo activation, an effect that is potentially associated through the induction of CD25+ Treg. These results may sustain the complex relationship between tumor growth and immune function where both the innate and adaptive immune responses are required for an effective anti-tumoral response, and cross-talk between these two arms of the response is essential.

**Tumor immunology**

Thus far cancer immunotherapy has been less successful than it was expected. Therefore, a step back is required to improve the understanding of the natural interactions between tumor and host IS. That is why tumor immunology has gained a renewed interest in recent years, in order to learn from nature. For the first time, the 6th edition of these international workshops organized by the CIM in Havana, devoted a full session to Tumor Immunology.

Tumor immunosurveillance theory is returning. Definitely the frequency of spontaneous and carcinogen-induced tumors is higher in genetically modified-immunodeficient mice. The central problem today is to understand how clinical tumors evade the immune response, and of course to define the available room for immunotherapeutic interventions in advanced cancer patients.

Two main hypothesis have been recently postulated to explain how tumors evade the immunosurveillance: tumor-induced immunosuppression and tumor immunoeediting. The clinical relevance of those phenomena is largely unknown. It was suggested by Rolando Pérez (CIM, Cuba), in his introductory remarks to this session, that the identification of key common targets for tumor progression and escape from the IS attack could be a novel potential approach to develop new therapies for cancer treatment.

Concerning tumor-induced immunosuppression, the role of Treg has been extensively documented, but recently the involvement of myeloid lineage derived cells in tumor-induced immunosuppression has been claimed. Some contributions at the meeting addressed this subject. Suzanne Ostrand-Rosenberg (University of Maryland, USA) showed that resistance to mammary metastatic disease in STAT6-/- mice (STAT6-deficiency prevents signaling trough the type 2 IL-4Ralpha) required CD8+ T cells, and is abrogated in STAT6/-IFN-gamma-/- double-deficient mice. A reduction in CD11b+Gr1+ myeloid suppressor cells (MSC) after primary tumor removal was found in STAT6-/- mice as compared to wild type BALB/c. In addition STAT6-/- mice produce type 1 (M1) macrophages, which contain high levels of nitric oxide (NO) and are tumoricidal for mammary carcinoma cells. Vincenzo Bronte (Padua University, Italy) followed a genome-wide profiling approach to study the physiology of CD11b+Gr1+ myeloid suppressor cells (MSC). The transcriptome analysis of CD11b+ cells enriched from spleen of tumor-bearing mice had an atypical granulocyte/inflammatory profile. Surprisingly, two enzymes, nitric oxide synthase 2 and arginase 1 were spontaneously upregulated during 24-hour cultures, suggesting that MSCs have properties of both M1 and M2-type macrophages, therefore to target L-arginine metabolism seems to be a novel potential strategy for cancer patient immunorestoration.

Tumor necrosis provides danger signals to activate natural anti-tumor immune response. But a remarkable signalling system for survival in hypoxia has been conserved throughout evolution, in which...
Attacking lymphocytes, or which trap lymphocytes. Humoral immune response to whereas CTL activity was only affected in MB16F10 models. In tumor-bearing mice cell-mediated immunity to OVA was impaired for CD4+ T cells, both sharing H-2K\textalpha subunit of the MHC class I antigen-processing machinery components in DC was significantly down-regulated by tumor-derived immunosuppressive factors, such as gangliosides. This includes LMP2, LMP7 and LMP10 (the subunits of the immunoproteasome). MB1 (β5, subunit of the constitutive proteasome), and the oxidoreductase ERp57. The role of the PDC, the main producer of the hypoxia inducible factor (HIF) plays a central role. Jacques Pouyssegur (Center Antoine Lacassagne, France) presented experiments of specific “silencing” of HIF 1α/2α inhibition by short interfering RNAs (siRNA) in normoxia. A sinergy in the HIF-1α activation process was observed in normoxia by “co-silencing” both, PHD2 and the HIF asparaginyl hydroxylase (FIH). In addition, he showed that vasoactive hormones (Ang II and thrombin) induce HIF-1α in vascular smooth muscle cells without affecting PHD2 levels. These signaling pathways that up-regulate Vascular Endothelial Growth Factor (VEGF) could have a strong impact on inflammation. Macrophages accumulate in hypoxic/necrotic tumor areas. Along with tumor cells, they also up-regulate HIFs 1 and 2, and paradoxically cooperate with tumor cells to ensure the vacularization of hypoxic areas, as explained by Claire Lewis (University of Sheffield, UK). This group proposed a novel approach based on the use of macrophages to carry therapeutic genes into the tumor. Autologous macrophages were transfected ex vivo with an adenovirus containing a transgene gene, the expression of which was regulated by a HIF-responsive promoter sequence.

Also the inhibition of the DC system by tumor derived factors has been well documented in both animal models and cancer patients. However, the mechanisms of poor antigen presentation by DC in cancer are largely unknown. Michael Shurin (University of Pittsburg, USA) demonstrated that expression of MHC class I antigen-processing machinery components in DC was significantly down-regulated by tumor-derived immunosuppressive factors, such as gangliosides. This includes LMP2, LMP7 and LMP10 (the subunits of the immunoproteasome). MB1 (β5, subunit of the constitutive proteasome), and the oxidoreductase ERp57. The role of the PDC, the main producer of type I IFN upon viral infection, in tumor biology is still unknown. Evelyn Hartmann (University of Munich, Germany) provided data demonstrating that high numbers of PDC infiltrate solid human primary tumor tissue. CpG-induced IFN\gamma production in tumor infiltrating-PDC was impaired. The expression of different TLR including TLR9 in PDC was down-regulated in the presence of tumor cells. These results suggest that tumor infiltrating-PDC may contribute to an impaired T cell-mediated response in tumors.

A novel approach to evaluate tumor induced-immunosuppression was proposed by Anabel de la Barrera (CIM, Cuba). She evaluated the immune response to vaccination with a neo-antigen in tumor-bearing mice. MB16 subclass 10 (MB16F10) and Lewis Lung carcinoma subclass D122 (3LL-D122), both sharing H-2K\textalpha haplotype, were used as tumor models. In tumor-bearing mice cell-mediated immunity to OVA was impaired for CD4+ T cells, whereas CTL activity was only affected in MB16F10 tumor-bearing mice. Humoral immune response to OVA was not affected.

Among the mechanisms developed by tumors to escape the IS attack, Kurt S. Zaneker (University Witten/Herdecke, Germany) proposed that highly motile cells shed membrane vesicles, which mislead attacking lymphocytes, or which trap lymphocytes in the connective tissue or cause signaling to lymphocytes to undergo apoptosis as shown by confocal microscopy. Both murine and human melanoma have been proven to express ectopically inhibitory FcyRIIB. Catherine Sautes Fridman (Center de Recherches Biomédicales des Cordeliers, France) showed that the ectopic expression of FcyRIIB on MB16F10 melanoma cells induced resistance of tumors to anti-gp75 mAb and to IgG anti-MB16F0 Ab raised in allogeneic BALB/c hosts. These results suggest that the ectopic expression of FcyRIIB on tumors may be a way of escaping humoral anti-tumor immunity.

Naren M. H. Ravindranath (University of Southern California, USA) compared the expression of three different complement restriction factors CRFs (CD46, CD55 and CD59) in different human tumor types. Breast and pancreatic cancer expressed a pattern of CRFs identical to that found in Oral Squamous Cell carcinoma (OSCC), suggesting that the microenvironment may be a main factor governing the expression of CRFs. Furthermore, he documented that IL-2 binds to IL-2 receptors on OSCC cells to induce the expression of CRFs, and to escape from the Ab mediated attack. One remarkable example for molecular mechanisms involved in tumor progression that could also be related to the acquired resistance to anti-tumor natural immune response was provided by Barbara Seliger (Martin Luther University Halle Wittenberg, Germany). First, she showed that Antigen Processing Machinery (APM) downregulation in tumors is most likely due to dysregulation rather than structural alterations. Sequence analysis of different components of the MHC class I antigen processing machinery in a large series of human and murine tumors revealed the occurrence of rare mutations in the peptide transporter subunits, in tapasin and in the low molecular weight proteins LMP2 and LMP7. Second, Her2 oncogen overexpression in tumor cells was correlated with a downregulation of APM.

Finally, Lina Matera (University of Turin, Italy) discussed how some oncospecific treatments could modify the anti-tumor natural immune response, by modifying tumor death rate, DC tumor uptake and cross-presentation of tumor associated antigens (TAA) to autologous T lymphocytes. Interestingly mAb-coated live tumor cells were taken up by DC much more efficiently than the necrotic or apoptotic ones and induced the highest DC alloantigen presentation and IL-12 release.

**Active specific immunotherapy**

The last session was devoted to Active Specific Immunotherapy (ASI) of malignant tumors. A new option of therapeutic modality, intending to mobilize the cancer patient’s own IS to recognize and destroy disseminating tumor cells, is now the most polemic and exciting variant of emerging treatments. Specificity of the effector arms of immunity, if properly activated, will assure the highest possible “benefit to risk” ratio for the patients. Since the clinical research introduction of ASI in the late 70s more than 400 cancer vaccine clinical trials have been conducted worldwide. Nevertheless, until now many complex factors that were properly discussed in the
preceding sessions have hampered the introduction of this concept in medical practice. In this session interesting clinical and pre-clinical applications on outstanding cellular vaccines and molecularly defined antigen vaccines (EGF, gangliosides, recombinant idiotypes, anti-idiotypic Ab, peptides and naked DNA) were introduced.

Dr. Donald Morton (John Wayne Cancer Institute, USA) discussed results from the largest AJCC stage IV melanoma study available in which Canvaxin (allogeneic melanoma cellular vaccine) treated patients survived almost twofold compared with untreated patients. Dr. Angus Dalgleish (St. George’s Hospital Medical School, UK) continued allogeneic cellular vaccine presentations with the Onyxvax prostate cancer vaccine. In an ongoing phase II clinical trial 42% of the patients had a greater than 30% reduction in the rate of rise of PSA, which was maintained and which correlated with time to progression, as measured by symptoms and then objective assessment. Dr. Senthamil Selvan (Hoag Cancer Center, USA) introduced a novel approach: the interferon-gamma symptoms and then objective assessment. Dr. Tania Crombet (CIM, Cuba) updated the results up of 8 months, progression-free survival is 51%.

Starting with the antigen-defined vaccine projects Dr. Tania Crombet (CIM, Cuba) updated the results of the ongoing randomized phase II trial in stage IIIb/IV NSCLC patients of active immunization with human recombinant EGF coupled with P64k protein from Neisseria meningitidis. Notably, a significant increase in survival was observed in vaccinated patients (median 8.0 months) while in the control group the median survival was 4.4 months. Returning to the polemic matter of gangliosides and cancer Dr. Beth Furst (St. George’s Hospital Medical School, London, UK) introduced the concept of ganglioside as tumor suppressor proteins. In summary this session constituted a review of modern trends in cancer immunotherapy with vaccines and several interesting ideas emerged that will soon impact in new strategies.