ISTITUTO SUPERIORE DI SANITÀ

International meeting on cancer vaccines

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ABSTRACT BOOK

Edited by
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The meeting is organized by the Istituto Superiore di Sanità (the Italian National Institute of Health) in collaboration with the National Cancer Institute of the US National Institutes of Health with the aim to give special attention to the recent progress in basic and applied research in the field of cancer vaccines. The meeting follows the first international Conference on Cancer Vaccines organized by the Istituto Superiore di Sanità in 1999. The major aims of the meeting are: i) to review the state of the art on the clinical research on cancer vaccines; ii) to present the new insights for designing therapeutic and preventive vaccines against tumors; iii) to illustrate new strategies for enhancing vaccine efficacy by the use of adjuvants, dendritic cells and combination therapies; iv) to present aspects and approaches relevant for the monitoring of the anti-tumor immune response.
Key words: Neoplasia, Immunotherapy

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Il convegno è organizzato dall'Istituto Superiore di Sanità in collaborazione con il National Cancer Institute degli US National Institutes of Health con l'obiettivo di dedicare una particolare attenzione ai recenti sviluppi della ricerca di base ed applicata nel campo dei vaccini antitumorali. Il convegno fa seguito alla prima Conferenza internazionale su vaccini antitumorali realizzata dall'Istituto Superiore di Sanità nel 1999. I principali obiettivi del convegno sono: i) offrire una rassegna dello "stato dell'arte" della ricerca clinica sui vaccini antitumorali; ii) presentare le nuove vedute per il disegno di vaccini preventivi e terapeutici contro i tumori; iii) illustrare nuove strategie per potenziare l'efficacia dei vaccini mediante l'uso di adiuvanti, cellule dendritiche e terapie combinate; iv) presentare aspetti rilevanti della risposta immune antitumorale e approcci per il suo monitoraggio.
Parole chiave: Neoplasia, Immunoterapia

Meeting organized by: Istituto Superiore di Sanità in collaboration with the National Cancer Institute of the US National Institutes of Health

Scientific Committee
E. Garaci (Honorary President), F. Belardelli, M. Ferrantini, G. Parmiani, J. Schlom

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Session I

Therapeutic vaccines against cancer: where are we now?

Chairpersons
E. Garaci, J. Harford

STATE OF THE ART ON THERAPEUTIC CANCER VACCINES

Giorgio Parmiani
Unit of Immunotherapy of Human Tumors, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.

During the last few years the results of several clinical studies of vaccination have been published. These can be considered as second generation of anti-cancer vaccines, considering the cell-based vaccines as those belonging to the first generation.

The clinical studies of the second generation are based on the use of peptide/proteins to construct vaccine aimed at testing in the clinics the principle that tumor-derived CD8 T cell epitopes given with traditional adjuvants (e.g. IFA), with cytokine-based adjuvants (e.g. GM-CSF) or loaded on autologous dendritic cells, could generate a T cell immune response targeting tumor cells and translating into a clinical response. Such studies will be summarized and discussed to identify strengths and weaknesses of those approaches. I will focus also on the many escape mechanisms that allow tumor cells to avoid destruction by immune T cell. Such an analysis should allow to design more effective clinical protocols in cancer vaccination.

INDUCTION OF NY-ESO-1-SPECIFIC IMMUNE RESPONSES IN CANCER PATIENTS

Alexander Knuth[1] and Elke Jäger[2]
[1]*University Hospital Zürich, Switzerland,* [2]*Krankenhaus Nordwest, Frankfurt, Germany.*

Cancer vaccines may have more favourable effects by stimulating integrated immune responses involving $CD4^+$ and $CD8^+$ T cell and B cell responses. To broaden the immunogenic profile of NY-ESO-1 vaccines to both MHC class I and class II restricted epitopes, recombinant vaccinia- and fowlpox NY-ESO-1 constructs were administered as a vaccine in a clinical study. Vaccinia- and fowlpox-NY-ESO-1 constructs used at 2 different dose levels were shown to be safe after intradermal and subcutaneous injection at monthly intervals for 4 months. Since NY-ESO-1 antibody negative, HLA-A2 positive patients were recruited for the first study cohorts, the HLA-A2 restricted NY-ESO-1 epitopes p157-167 and p157-165 were used for DTH testing and to monitor $CD8^+$ T cell responses during the course of immunization. Twelve HLA-A2 positive patients were enrolled, 7 have completed 4 immunizations. Peptide-specific $CD8^+$ T cell responses were induced in all 7 patients who completed the protocol. The induction of NY-ESO-1 antibody was observed in 1 patient after 2 immunizations. There was no evidence of disease progression in 6 patients for > 6 months after the start of immunization. Additonal analyses for the identification of $CD4^+$ and $CD8^+$ T cell responses directed against other NY-ESO-1 epitopes are ongoing in different international collaborative projects within the Ludwig Institute. The results will contribute important information on the efficacy of recombinant viral NY-ESO-1 constructs in inducing integrated NY-ESO-1-specific immune responses involving $CD4^+$, $CD8^+$ T cells, and antibody, and their impact on the clinical development of NY-ESO-1^+ disease.

VACCINATION OF RECONSTITUTED LYMPHOPENIC HOSTS WITH GENETICALLY MODIFIED TUMOR CELLS GENERATES T CELLS FOR EFFECTIVE ADOPTIVE IMMUNOTHERAPY

Bernard A. Fox[1] Christian H. Poehlein[1], Jun Ma[2], Shawn M. Jensen[1], Michael G. LaCelle[1], Dan Haley[1], Hong-Ming Hu[2], Brendan Curti[1], Dominik Ruettinger[3], Tarsem Moudgil[1], Natasja van den Engel[3], Hauke Winter[3], Rudolf Hatz[3], Yili Wang[2], Edwin B. Walker[1] and Walter J. Urba[1]

[1]Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center and Departments of Environmental and Biomolecular Systems, and Molecular Microbiology and Immunology, OHSU, Portland, Oregon, USA, 97213; [2]Institute for Cancer Research, Xi'an Jiaotong University, Xi'an, China; [3]Department of Surgery, Klinikum Grosshadern, LMU, Munich, Germany.

Vaccination strategies have failed to significantly impact outcomes of cancer patients. We hypothesize that a primary reason for this failure is because the magnitude of the antitumor 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 spleen cells. Tumor vaccine-draining lymph nodes (TVDLN) of reconstituted lymphopenic mice (RLM), vaccinated with a GM-CSF-secreting tumor vaccine, contained an increased number of activated T cells tumor-specific CD4 and $CD8^+$ T cells. Following *in vitro* activation and expansion TVDLN T cells from RLM were significantly ($p<0.05$) more therapeutic in adoptive transfer studies than TVDLN T cells from normal «intact» mice. Additional studies documented that this strategy was also highly effective in an active-specific immunotherapy model. Recent studies have examined how the degree of lymphopenia affects the developing immune response and what effect a pre-existing tumor-bearing state has on this strategy. Based on these data we are initiating trials of this strategy in patients with prostate, melanoma, ovarian and NSCLC.

This study was supported by grants from the Chiles Foundation, the M.J. Murdock Charitable Trust, NIH RO1CA80964 (BF), DOD PC001228 (BF) and PC020094 (BF)

Session II
Cancer vaccines and combination therapies

Chairpersons
A.L. Goldstein, M. Roselli

RECOMBINANT CANCER VACCINES AND COMBINATION THERAPIES

Jeffrey Schlom
Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, USA.

We have developed and/or employed multiple strategies to enhance T-cell responses directed against tumor-associated antigens (TAA). These are: (a) the use of viral vectors to deliver both TAA and immunostimulatory molecule gene products; (b) diversified prime and boost vaccination regimens employing recombinant vaccinia as a primary vaccination followed by multiple boosting vaccinations with replication defective avipox vaccine; (c) the use of viral vectors containing transgenes for a triad of T-cell costimulatory molecules (designated TRICOM); (d) altering the amino acid sequence of the TAA to enhance immunogenicity; and (e) the use of cytokines such as GM-CSF.

Clinical trials are ongoing employing vaccines directed against CEA, MUC-1 and PSA. Preclinical studies have emphasized the use of transgenic (Tg) mice. The vaccination of CEA Tg mice with CEA-TRICOM vectors induces higher levels of anti-tumor activity than vectors devoid of TRICOM. Recent findings in this preclinical model have demonstrated that local external beam irradiation of tumor in situ, at doses insufficient to reduce the rate of tumor growth, leads to phenotypic alterations of tumor cells (including upregulation of Fas) that make them more susceptible to specific immune attack. Tumor radiation and CEA-TRICOM vaccine when used together were shown to act synergistically in inducing anti-tumor responses. The mechanism involved in tumor destruction was defined as Fas mediated. In a double Tg model (APC min x CEA Tg) in which mice develop spontaneous CEA-expressing tumors (colonic polyps), CEA-TRICOM vaccine was shown to vastly reduce the development of spontaneous tumors. Moreover, the combined use of a COX-2 inhibitor plus vaccine was shown to further inhibit tumor development.

Phase I/II trials in patients with advanced CEA-expressing tumors, employing either viral vector-based vaccines or agonist peptide-pulsed dendritic cell vaccines, have demonstrated objective clinical responses, drops in serum markers, and T-cell responses specific for CEA. Moreover, preliminary data indicate a correlation between increased survival and the generation of CEA-specific T-cell responses. However, more comprehensive clinical studies are required and are currently ongoing or planned. These studies thus form a rational basis for the combinatorial use of recombinant vaccines with other forms of anti-cancer therapy.

BIOLOGICAL BASES OF COMBINATION THERAPIES

Enrico Garaci
Istituto Superiore di Sanità, Rome, Italy.

Multiple approaches of cancer immunotherapy have been tested in different experimental tumor models and in human cancers with variable success. Most of them are based on the hypothesis that the inhibition of tumor growth requires a strong immune response, in which a main role is played by CTLs. It is known, however, that an efficient CTL response requires expression of tumor antigens, MHC class I surface molecules presentation, expression of different costimulatory molecules and a sustained generation and proliferation of specific $CD8^+$ cells with an efficient $CD4^+$ cell cooperation. Over the last decade, our group has extensively tested a protocol of combined therapy consisting in the use of chemotherapeutic agents associated with thymosin alpha 1 (Talpha 1) and different cytokines (especially IFN-alpha and IL-2), showing a remarkable antitumor effect in response to this combined therapeutic regimen. Recent studies have revealed some major mechanisms by which certain myelotoxic agents, including cyclophosphamide, can induce a marked enhancement of the response to immunotherapy. On the other hand, recent studies have also described new effects of Talpha 1 and certain cytokines on cells of the immune system, including dendritic cells, which may be relevant for their use in combination therapies.

Thus, the recent research progress now offers new opportunities for the design of more selective and potentially effective strategies of combination of chemotherapy and immunotherapy, which might result in a major advance in the treatment of human cancer.

PRECLINICAL MODEL OF COLORECTAL CANCER VACCINE: A MODEL FOR A COMBINED APPROACH

G. Rasi
Institute of Neurobiology and Molecular Medicine, Italian National Research Council (CNR) Rome, Italy.

Development of Immunotherapy basically consists in the induction of a specific CTL response against one or more targets on the tumor cells. This nowadays could be reached by many means, immunizing with peptides or DNA and using APC and/or cytokines to rise or boost the immune response. Despite the encouraging and continuously improving results the outcomes are inconstant and not universally applicable.

Some issues still should be addressed to further improve the effects of immunotherapy: obtain a CTL response sustained in time and intensity, verify that the tumor remain "visible" to the immunesystem for a sufficient time and identify new and more selective tumor targets. These could be achieved only with a combined strategy instead of a single approach.

We developed an experimental model of liver metastases from colorectal obtained injecting DHD-K12 tumor cells in the splenic vein of syngeneic BDIX rats. Some important characteristics make this model of particular interest for cancer vaccine development: the tumor naturally expresses at least three conserved tumor antigens identical to those of human colorectal cancer, liver metastases growth could be followed by laparotomy or MRI, immunerersponse could be followed by repeated peripheral blood drawing, drugs and fluids could be administered either systemically or locoregionally as a continuous infusion by positioning an osmotic minipump in the peritoneal cavity.

Specifically in this model were demonstrated: the efficacy of a peptide vaccine (immunizing the rats with the nonapeptide RTKNEASIC) in preventing liver metastases, the efficacy of a naked DNA vaccine in reducing the tumor growth, the possibility to up-regulate the expression of molecular target *in vivo* by systemic treatment (with chemotherapeutic agents and/or Tα1), the efficacy of combined chemo-immunotherapy using 5-FU, IL-2 and Tα1. The possibility to further expand this model in order to achieve information useful for the clinical setting of cancer vaccine strategies will be discussed.

CHEMOTHERAPY: FRIEND OR FOE TO CANCER VACCINES?

Leisha A. Emens
The Johns Hopkins University School of Medicine, Baltimore, MD, USA.

Despite significant progress in the development of cancer vaccines, tumor-specific immune tolerance and the magnitude of tumor burden remain two major obstacles to their clinical efficacy. Combinatorial vaccination strategies that integrate tumor vaccines with traditional and novel cancer therapeutics in order to minimize tumor burden and circumvent established mechanisms of immune tolerance are thus a high priority for preclinical development. Neu-N transgenic mice represent a clinically relevant preclinical model, since they spontaneously develop neu-expressing mammary carcinomas in the setting of a profound, pre-existing neu-specific immune tolerance to the endogenously expressed rat neu protein. We have recently demonstrated that nontolerized parental FVB/N mice reject large burdens of pre-established neu-expressing tumors after vaccination with granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting neu-specific cellular vaccines. Tumor rejection is associated with the induction of a high avidity $CD8^+$ T cell response almost exclusively specific for the immunodominant neu epitope RNEU420-429 (p50). In contrast, vaccinated tolerogenic neu transgenic mice develop a heterogeneous, low avidity neu-specific $CD8^+$ T cell population characterized by a paucity of p50-specific $CD8^+$ T cells. Strikingly, we have also shown that chemotherapy-modulated vaccination, with Cyclophosphamide given at the time of T cell priming and Doxorubicin given at the time of T cell expansion, can induce a curative high avidity p50-specific $CD8^+$ T cell response in about 30% of treated neu mice. We have also shown that low dose Cyclophosphamide augments vaccine-activated immunity in neu mice by abrogating the suppression of high avidity p50-specific $CD8^+$ T cells by $CD4^+CD25^+$ T regulatory cells. Thus, timed sequential therapy with low dose chemotherapy and a GM-CSF-secreting vaccine can overcome at least one mechanism of antigen-specific immune tolerance. These studies lay the groundwork for a clinical trial testing chemotherapy-modulated vaccination in patients with metastatic breast cancer.

IRX-2 IMMUNOTHERAPY FOR PATIENTS WITH SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

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Introduction: To evaluate clinical responses and long-term recurrence free survival (RFS) in patients treated with combination immunotherapy using naturally derived TH1 cytokines (IRX-2).

Method: 32 advanced, operable H&N SCC patients were treated for 21 days with the IRX-2 protocol (single infusion of low dose cyclophosphamide (300 mg/M2), 10 or 20 day perilymphatic injections of IRX-2 at low doses (200 units IL-2 equivalence) in the neck and daily oral indomethacin and zinc) and compared with 28 site and stage matched concurrent institutional controls. All patients were treated with surgery and radiotherapy if indicated.

Results: No significant IRX-2 toxicity was observed. IRX-2 treated patients showed reduction of symptoms. Fifteen patients had CR/PR (3/12) and 3 had minor responses (comparing pre-surgical staging with the surgical specimen). Histological sections of the surgical specimens showed in all 32 IRX-2 treated patients further tumor reduction (39%), with tumor fragmentation and increased leukocyte infiltration, mainly lymphocytes, in contrast to none of the controls. At 48 months of follow up the IRX-2 treated patients had RFS of 69% in contrast to the controls 28% (p=.002). The 20-day injection protocol differed from the 10-day injection protocol only in having less leukocyte infiltration in the surgical specimen. Both clinical and histological tumor responses correlated with increased RFS (p's<.01). The lymph nodes of patients with H&N SCC are distinguished by T cell depletion and sinus histiocytosis. Immunotherapy reverses these changes and induces nodal expansion with tumor lymphoid infiltration into the tumor that correlates with LN changes.

Conclusion: This phase II study documents the ability of IRX-2 immunotherapy to induce lymphocyte mobilization and tumor infiltration, and to lead to immune regression and improved survival of H&N cancer patients. The correlation of nodal expansion with tumor lymphoid infiltration and regression implies an effective immunization to host tumor antigens occurring at the level of the regional lymph node. The reversal of sinus histiocytosis, by IRX-2 treatment, in association with nodal expansion suggests that tumor antigen processing via dendritic cells is defective in cancer-bearing patients and that it is corrected by the IRX-2 immunotherapy treatment.

VISUALIZATION OF TUMOR ANTIGEN SPECIFIC CD4$^+$ T CELL SUPPRESSION

Hyam I. Levitsky and Gang Zhou
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There is now ample evidence that tumors arising in immunocompetent individuals frequently express antigens that are recognizable by host T cells. The impact of tumor progression on the function of tumor-antigen specific T cells has been examined in a number of experimental model systems that have demonstrated diverse outcomes ranging from T cell ignorance to induction of effector function to the development of anergy. Using the adoptive transfer of CD4$^+$ T cells from TCR transgenic mice recognizing an MHC class II restricted epitope of influenza hemagglutinin (HA) into mice harboring a systemic B cell lymphoma expressing HA (A20HA), we find clear evidence of antigen recognition. Clonotype$^+$ T cells undergo a modest expansion, and display a cell surface phenotype consistent with antigen experienced T cells. Paradoxically, analysis of cell division by CFSE dilution reveals that only a minority of the HA-specific T cells enter the cell cycle, in spite of a progressively expanding systemic tumor burden and increased availability of the nominal antigen. 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 & IL-2 production), the divided cells have 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 profiling of CFSE$_{lo}$ 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 CFSE$_{lo}$ 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, whereas the undivided HA-specific T cells from A20HA bearing mice did not suppress. 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. Transwell experiments demonstrated the requirement for cell:cell contact for suppression. Suppression was antigen-specific, as mixing CFSE$_{lo}$ 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 CFSE$_{lo}$ 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 (i.e. clonal expansion) of the responder T cells and blocked their differentiation into INF-γ producing Th1 cells. Studies examining the mechanism of suppression by tumor-antigen experienced $CD4^+$ T cells, their lineage, and factors required for their differentiation are underway.

IMMUNOTHERAPY-INDUCED TUMOR REGRESSION IN MICE TREATED WITH MYELOTOXIC DRUGS: NEW RATIONALES AND PERSPECTIVES OF CLINICAL APPLICATIONS

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Few cases of clinical response have been observed in patients with advanced malignant diseases treated with cancer vaccines, even though an enhancement in the number of circulating tumor-specific T lymphocytes has often been detected. It has been suggested that defects in T cell functions may be responsible for the poor response. We reasoned that the adoptive transfer of tumor specific T cells could overcome the tumor-induced immune suppression, provided that the pre-existing tolerogenic immune response is erased by mean of cytotoxic drugs. Recently, we have demonstrated a remarkably positive influence of chemotherapy and radiotherapy treatments on the activation/expansion and antitumor activity of adoptively transferred tumor-specific T cells in different mouse tumor models. In particular, we have shown that a non-myeloablative dose of cyclophosphamide (CTX) or a gamma ray sub lethal irradiation, administered before immune cell transfer, completely cured mice bearing established metastatic tumors. Unlike the current view of a dominant role of $CD8^+$ lymphocytes, the immune cells responsible of the transfer of the antitumor immunity were mostly $CD4^+$ T cells. Notably, combined CTX treatment and adoptive cell therapy were ineffective in tumor-bearing SCID mice, indicating the need of cooperation between transferred and host cells. Transferred cells underwent *in vivo* expansion only after CTX administration and only lymphocytes derived from immunized donors homed to the tumor tissue. Non myeloablative CTX doses proved essential to promote homeostatic proliferation and activation of transferred immune lymphocytes. We could also show that a similar combined therapy was even capable of eradicating the chronic infection of mice with the MHV-68, a model closely resembling the latent EBV infection in humans. With the aim of exploiting the same mechanism of action of CTX on the immune system, we recently treated tumor-bearing mice with tumor lysates shortly after CTX administration (at the time of "cytokine storm", i.e. when a broad array of cytokines are induced by the drug) and could detect an impressive antitumor response only in animals subjected to the combined therapy. In the light of these preclinical data, we have designed a clinical trial, where stage II-IV melanoma patients will be vaccinated with melanoma peptides while undergoing dacarbazine chemotherapeutic regimen. Thus, new rationales are now available for combining immunotherapy and chemotherapy, which could result in the development of highly effective novel strategies for inducing clinical response in patients with cancer and certain chronic infectious diseases

RATIONALE USE OF CHEMOIMMUNOTHERAPY OF COLON CARCINOMA

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The possibility to combine chemo and immunotherapy for colon cancer is an attractive modality to improve the effectiveness of both strategies. The chemotherapy may in fact produce a rapid tumor debulking, a change of tumour cell phenotype resulting in the up-regulation of potential cancer vaccine targets and may affect tumour cell resistance to death signals utilized by the vaccine-activated effectors. We have shown that a chemotherapy regimen with multiple cytotoxic drugs including 5-FU, enhances colon cancer cell immunogenicity, which became useful to prime an efficient tumour-specific CTL immune reaction. Human CTL lines generated by cross-priming DC with chemotherapy treated colon carcinoma cells to stimulate autologous PBMC of normal donors and patients with colon carcinoma showed greater anti-tumour activity against colon carcinoma cells *in vitro* and contained subsets of effector T cells with higher ability in recognising TS and CEA. We have also conducted a Phase II trial in colon carcinoma patients in first–second line of treatment, receiving sequential treatment with gemcitabine, oxaliplatin, leucovorin and 5-FU (GOLF) followed by daily sc administration of GM-CSF and IL-2, reporting in the first 20 enrolled individuals an unusual high rate of response (80%) with low toxicity. An immunological study of these patients revealed that the treatment induced a proliferative T cell response to colon cancer antigens and produced an enhanced CTLs with high anti-tumour activity and TCR avidity against known HLA-A(*)02.01 binding epitopes of CEA and TS. Our data suggest that a rationale combination of chemo- and immunotherapy could result in the generation of multi-antigen specific CTLs with anti-tumour activity, and could be an attractive strategy to be investigated in future clinical trials.

Session III
Adjuvants for the development of cancer vaccines

Chairpersons
H.C. Morse, I. Gresser

ADJUVANTS, CYTOKINES AND VACCINE DEVELOPMENT

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The word “adjuvant” is derived from the Latin word “adiuvare” which means “to help”. For immunologists, it defines substances capable of helping the generation of the immune response to antigens. Today, the identification of new adjuvants can be considered as a fundamental step in vaccine development. In fact, while a remarkable progress has been achieved in the identification of molecular components of pathogens and in the characterization of new TAAs, the research on adjuvants has progressed slowly. Alum, the most currently used compound in human vaccines, is a weak adjuvant; very few other adjuvants have been used in humans and safety concerns often restrict their clinical use. Recently, synthetic CpG oligonucleotides have been considered as potential adjuvants on the basis of results of pilot clinical trials. Only recently, have we begun to understand the mechanisms of action of some of these adjuvants and their action on dendritic cells (DCs), which is essential for induction of protective immunity. Some cytokines may act as vaccine adjuvants. A considerable attention has been given to GM-CSF, but no general consensus exists on the efficacy of this cytokine as vaccine adjuvant. Local administration of IL-12 has been reported to function as an adjuvant of cancer vaccines. Our group has recently demonstrated that type I IFN, cytokines with a long record of clinical use, act as powerful vaccine adjuvants in mouse models and are important factors for the differentiation/activation of DCs; clinical studies aimed at evaluating the adjuvant activity of IFN-alpha in vaccination strategies with the HBV vaccine in healthy subjects and with MART-1 and gp100 peptides in melanoma patients have recently been activated. Traditionally, successful vaccines have been generated largely against pathogens causing self-limiting infections and consisted of live attenuated pathogens; thus, the paradigm has been to attempt to mimic natural infection. Under these conditions, infection-induced danger signals, including IFNs, can play an important role in the generation of protective immunity. As the current trend is now the use of subunit vaccines (which are safer, but poorly immunogenic), the identification of more effective adjuvants turns out to be essential for vaccine development. Powerful adjuvants are especially needed for the development of cancer vaccines, as they should be capable of breaking tolerance towards self TAAs and of counteracting tumor-induced immune-suppression. The recent progress in immunology has just started to teach us how to use nature’s adjuvants, such as DCs and certain cytokines, for the development of human vaccines. Information stemming from the research progress in basic and clinical immunology and on new adjuvants will be instrumental for the development of preventive and therapeutic vaccines against some life threatening diseases, including cancer.

ENHANCING CANCER VACCINES BY *IN VIVO* ACTIVATION OF TLR9 WITH CPG OLIGOS

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Unmethylated CpG dinucleotides in bacterial or viral DNA stimulate innate and adaptive immunity by engaging Toll-like receptor (TLR)9. These immune effects can be mimicked by synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN). In humans the only immune cells that express TLR9 and are activated directly by CpG ODN are B cells and plasmacytoid dendritic cells (pDC). Depending on the structure and sequence of the CpG ODN, at least three distinct classes of immune effects can be induced. Depending on their class, CpG ODN activate B cells to proliferate and secrete immunoglobulin; and pDC to secrete a variety of Th1-like cytokines, chemokines, and type I interferons, and to express increased costimulatory molecules. When activated by CpG, pDC gain the ability to stimulate Th1-like T cell responses. CpG ODN induce immune defense mechanisms that protect against challenge with a wide range of infectious pathogens, and have shown therapeutic activity in animal models of allergic disease and cancer. CpG ODN are also extremely effective vaccine adjuvants, inducing Th1 responses in mice and primates with both specific antibody and CTL. As an adjuvant for a hepatitis B vaccine, a B-Class ODN, CpG 7909, appears to induce earlier seroconversion with the production of increased levels of specific antibody and cellular responses. A phase II human clinical trial of CpG 7909 in HIV-infected subjects, including prior hepatitis B vaccine nonresponders, showed a striking increase in the magnitude of antibody responses in the subjects receiving CpG as a vaccine adjuvant. CpG ODN are produced by cGMP chemical synthesis, have excellent stability and solubility, and show an excellent safety profile, suggesting wide utility as vaccine adjuvants. In phase I studies, CpG 7909 has shown promising activity as an adjuvant for cancer vaccines. CpG 7909 also has shown activity as a monotherapy in phase I clinical trials in basal cell carcinoma, cutaneous T cell lymphoma, melanoma, non-Hodgkin's lymphoma, and renal cell carcinoma. CpG 7909 is now in phase II clinical trials in combination with chemotherapy for lung cancer and melanoma. A C-Class oligo, 10101, has recently entered human clinical trials as a monotherapy for chronic hepatitis C virus infection.

CLINICAL USE OF IL-12 AS AN ADJUVANT FOR CANCER VACCINES

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Preclinical data have suggested that a Th1/Tc1-directed immune response may be optimal for tumor rejection *in vivo*. Interleukin-12 (IL-12) is one key factor that promotes Th1/Tc1 T cell differentiation, which has led to the integration of this cytokine into therapeutic vaccine strategies. In murine studies, immunization with IL-12-transfected tumor cells, rIL-12 combined with antigenic peptides in adjuvant or peptide-pulsed antigen presenting cells (APCs), or IL-12 engineered into viral constructs have shown improved immune responses and tumor rejection. These encouraging data have led to several clinical trials of vaccines incorporating IL-12. We have observed that immunization with MelanA peptide-pulsed PBMC + rhIL-12 induces T cell responses and clinical activity in HLA-$A2^{+}$ patients with advanced melanoma. A post-surgical adjuvant trial in melanoma has shown augmented T cell responses with IL-12 added to peptides emulsified in Montanide adjuvant. Alternative vaccine adjuvants may elicit endogenous IL-12 production from host cells. Despite induction of a tumor antigen-specific Th1/Tc1 immune response in many patients, tumor progression often occurs. These results point towards mechanisms of tumor resistance downstream from initial T cell priming. Current research efforts are focusing on candidate mechanisms of resistance, including T cell anergy, expression of inhibitory ligands such as PD-L1/B7-H1, and metabolic limitations of the tumor microenvironment.

Session IV

Human tumor associated antigens and cancer vaccines

Chairpersons
P.G. Natali, R. Foà

NEW PERSPECTIVES ON TUMOR REJECTION RESPONSES AFTER PEPTIDE VACCINATION

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Antigens encoded by gene MAGE-3 have been used for small-scale therapeutic vaccination trials of metastatic melanoma patients, either as antigenic peptides, protein, a pox family recombinant virus carrying a MAGE-3 sequence, or peptide-pulsed dendritic cells. Clinical responses were observed in 10-20% of the patients. To detect anti-MAGE-3 T cell responses we resorted to an *in vitro* restimulation with antigenic peptide before labeling with tetramers. In order to evaluate precursor frequencies, these cultures were carried out in limiting dilution condition. Tetramer-positive cells were sorted and cloned. The specificity of the CTL clones was verified, and their TCR sequenced. We found a CTL response in 5 out of 10 regressor patients and in 2 out of 18 progressors, suggesting a correlation between the occurrence of these CTL responses and the tumor regressions. In addition, we wished to examine whether T cells recognizing other tumor antigens might contribute to the tumor regressions. We used autologous melanoma lines from 6 vaccinated patients to estimate blood frequencies of anti-tumor CTL, namely lytic effectors that recognized autologous tumor cells but not autologous B cells or NK targets. After vaccination, frequencies of anti-tumor CTL ranged from 10^{-4} to 3 x 10^{-3} of the blood CD8 T cells, i.e. 10 to 10,000-fold higher than those of the anti-vaccine CTL in the same patients. Frequencies of similar magnitude were already present prior to vaccination. We were able to identify the antigens recognized by 13 out of 15 anti-tumor CTL clones derived from a patient who had shown nearly complete tumor regression following vaccination. Ten CTL clones recognized antigens encoded by the cancer-germline gene MAGE-C2, and 2 recognized antigens encoded by the melanocyte differentiation gene gp100. A CTL clone recognizing a MAGE-C2 antigen was present in the blood at a freqeuncy of 9 x 10-5 and in an invaded lymph node at more than 9 x 10^{-2}. Other anti-tumor CTL were also highly enriched in tumor deposites. These results suggest that anti-vaccine CTL may exert their main effect by triggering in the tumor a stimulation of other anti-tumor CTL which destroy the tumor cells.

IMMUNOLOGICAL AND CLINICAL EFFECTS OF VACCINATION WITH AUTOLOGOUS TUMOR-DERIVED HSP96

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With the aim of identifying more effective vaccine strategies in cancer patients, we investigated the role of human tumor-derived HSP-gp96 in modulating anti-tumor immune reactivities both *in vitro* and *in vivo*. The ability of gp96 to chaperone immunogenic peptides was assessed by using an immunological approach, evaluating the ability of tumor-derived gp96 to reconstitute the epitopes recognized by $CD8^+$ T cells specific for highly expressed melanoma or colon carcinoma antigens. These experiments showed that $CD8^+$ T cells recognizing antigens expressed in human melanoma (MART-1/Melan-A) or colon carcinoma (CEA and EpCAM) were triggered to release IFN-γ and to mediate cytotoxic activity by HLA-matched APCs pulsed with gp96 purified from tumor cells expressing the relevant antigen. Such activation occurred in class I HLA-restricted fashion and was more efficient than that achieved by direct peptide loading.

The immunological monitoring of melanoma and colon carcinoma patients vaccinated with autologous tumor-derived gp96 showed that activation and expansion of tumor-reactive $CD8^+$ T cells occurred in approximately 50% patients, as detected by IFN-γ Elispot. Additionally, a boost of MART-1/Melan-A_{27-35}, and of $CEA_{571-579}$ and $EpCAM_{263-271}$ peptide recognition was observed in a subset of HLA-A2 melanoma and colon carcinoma patients, respectively. These increments in tumor and antigen-specific T cell responses were associated with a favorable disease course after gp96 vaccination. In addition to the effects on specific T cell-mediated anti-tumor responses, gp96 vaccine induced a significant increase of NK activity, associated to a raise of $CD16^+CD56^+$ and $CD3^+CD56^+$ lymphocytes and enhanced expression of the cytotoxicity receptors NKG2D and NK-p46 in these cell subpopulations. Experiments aimed at elucidating the potential mechanisms of gp96-mediated NK activation showed that this protein could bind to a subset of NK and activated $CD3^+$ T cells, trigger cytotoxicity and cytokine-release and promote increased activation marker expression. Additionally, gp96-vaccinated patients diplayed up-regulation of CD83 and CD80, and enhanced IL-12 release in $CD14^+$ monocyte cells, suggesting that NK activation could also occur through a monocyte-mediated indirect mechanism. Altogether these data suggest that gp96 mediates pleiotropic anti-tumor responses involving both adaptive and innate immunity, and may thus represent a valid tool of vaccine therapy in cancer patients.

HMENA, A CYTOSKELETON REGULATORY PROTEIN OVEREXPRESSED IN BREAST CANCER ELICITING BOTH HUMORAL AND CD8$^+$ T CELL IMMUNE RESPONSE

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We have recently identified through the SEREX approach in breast cancer hMena, as the human ortholog of murine Mena protein belonging to the ENA/VASP family of protein which modulate actin cytoskeleton dynamics often deregulated in tumors. Following cloning and sequencing three hMena isoforms were identified and we investigated their expression by Western Blot in a large panel of tumor cell lines of different histotypes and in a number of normal cells. We demonstrated that hMena is overexpressed in a high percentage of tumors with respect to the normal cells and that the isoforms are differently modulated in epithelial or mesenchymal cells.

In breast cancer we analyzed hMena expression by immunohistochemistry in a representative panel of benign, preneoplastic and neoplastic lesions. hMena, while undetectable in normal mammary epithelium and in benign lesions, is consistently overexpressed in tumors and in preneoplastic lesions at high risk of transformation, thus suggesting that hMena overexpression is an early event in breast tumorigenesis.

A cancer-restricted antibody response against hMena was demonstrated and we have identified three hMena peptides representing HLA-A2 restricted T cell epitopes recognized by CD8$^+$ T lymphocytes of HLA-A2 breast cancer patients, as evaluated by ex vivo IFNγ ELISPOT assay. This spontaneous CD8$^+$ T cell response was in some instances concomitant with the antibody response in breast cancer patients, bearing hMena$^+$ tumors. Furthermore, we established hMena specific T cell lines from different HLA-A2 positive patients and functional studies have demonstrated that at least one of the hMena peptides identified (hMena-502) is naturally processed in a breast cancer cell line as well as in a melanoma cell line.

hMena promises to be an attractive model for exploring breast tumorigenesis and the kinetics of the correlated immune response, contributing to new insights in breast cancer biology and management.

IMMUNOTHERAPY OF MELANOMA USING A UV INACTIVATED VACCINIA VIRUS EXPRESSING MULTIPLE EPITOPES AND COSTIMULATORY MOLECULES

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A phaseI/II clinical trial in metastatic melanoma patients was performed with a UV-inactivated recombinant vaccinia virus expressing endoplasmic reticulum (ER) targeted HLA-A0201 restricted Melan-A/Mart-1 27-35, GP100 280-288 and tyrosinase 1-9 epitopes, together with CD80 and CD86 costimulatory proteins. Corresponding soluble peptides were used to boost responses and GM-CSF as systemic adjuvant.

Beside a single transient grade 3 leukopenia, no major clinical toxicity was reported. Immunogenicity, as monitored on *in vitro* restimulated PBMC by CTL precursor (CTLp) frequency analysis and tetramer staining could specifically be addressed in 18 patients (stage III, n=5; stage IV, n=13). Increases (at least two fold) in specific CTLp frequencies were observed in 15. Responsiveness against all 3 antigens could be analyzed in 16 patients: 7 (43%), including all stage III cases, showed evidence of induction of CTL specific for the three epitopes, 2 (12%) and 4 (25%) respectively, showed reactivity against two or one TAA. In 3 stage IV patients no specific CTL reactivity could be induced. Remarkably, increase in CTLp frequency, as well as increase of Mart-tetramer positive CD8+ cells, were mostly detected after viral vaccine injections. However, in a majority of patients, CTLp levels upon completion of protocol were comparable to levels prior vaccination. On the other hand, patients displaying vaccinia virus specific humoral responses, evaluated by ELISA, were able to generate a TAA specific CTL induction.

This is the first report on the administration in melanoma patients of a UV-inactivated recombinant vaccinia virus co-expressing 5 transgenes. The results described here, in terms of safety and detectable immunogenicity, support further investigation with such reagents in active specific immunotherapy.

Session V
Towards the development of preventive cancer vaccines

Chairpersons
G. Rezza, S. Vella

WHAT'S UP IN HIV VACCINES - ANY IMPLICATIONS TO CANCER

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Human retroviruses enhance the development of malignancies by both direct (HTLV-1) and indirect (HIV) mechanisms. HTLV-1 has multiple mechanisms for promoting T-cell proliferation. Presumably because some of these mechanisms lead to genetic instability, one or more infected cells may become transformed beginning the clonal neoplastic process. In contrast, the first documented role of an indirect "cause" of a malignancy is HIV infection in that the HIV infected cell does not become neoplastic. Rather, other uninfected cells do, and in untreated HIV infected patients malignancies arise at an amazingly high incidence (over 30%), making HIV-l one of the most effective human co-carcinogens. With the exception of some of the B-cell lymphomas, the malignancies which are AIDS associated are those harboring known human tumor viruses. Needless to say, development of a preventive vaccine against HIV is one of the greatest needs in biomedical science.

For obvious reasons all HIV vaccines now under test use HIV subunit vaccines. Early attempts were with the envelope gp120, aimed at inducing neutralizing antibodies (NA) which could block virus entry and thereby possibly achieve complete protection from infection, failed because the NA were not sufficiently broad. By the 1990's the field abruptly changed its objectives toward inducing CMI which do not give protection in monkey models but keep virus at low levels. This prompted trials (now on-going) in humans. Two arguments have been presented for this approach: (1) development of past successful vaccines did not achieve complete protection, e.g., polio; (2) the goal of obtaining broadly reactive sufficiently long lasting NA presumably is not reachable. Both arguments are invalid. Polio is usually ultimately eliminated, whereas with a retrovirus integration of its genes into target cells means that cells may later express virus and at levels which are unpredictable. Indeed, the earlier excitement about CMI vaccines has dwindled because robust replication of virus eventually occurred. As to(2): the recent advances in our knowledge of HIV entry have led to some novel results, namely and for the first time, broadly reactive NA against primary isolates of HIV from various clades have been obtained at our Institute (A. DeVico, T. Fouts and G. Lewis). I will describe the approach and the laboratory results. Finally, from HIV pathogenesis studies have led us to also target the HIV Tat protein, an essential regulator of HIV infection in infected cells, but also extracellular Tat exerts an effect on uninfected cells which contributes to immune suppression. This seems analogous to peptides released by tumor cells (e.g. E7 in HPV induced cervical cancer). The mechanism for this effect of Tat will be summaried. D. Zagury (Paris) and I have proposed adding a Tat vaccine to our main immunogen (see above) for prevention and Tat alone as a therapeutic vaccine.

PAPILLOMAVIRUS AND CERVICAL CANCER: PREVENTIVE VACCINES ARE ON THEIR WAY

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Every year approximately half a million women world-wide develop cervical cancer; 80% of them live in poor countries where population-based screening programmes are virtually non-existent. The role of sexually transmitted agents in the aetiology of cervical cancer has been suspected for more than a century, but knowledge in this field has rapidly expanded only in the last 20 years, after major improvements were made in detection methods for human papillomavirus (HPV).

A dozen types of HPV have been identified in 99% of biopsy specimens from cervical cancer world-wide and the relative risk (RR) estimates for HPV in case-control studies of cervical cancer range from 50 to 100. There is no effective medical treatment for HPV, but a prophylactic vaccine, based on L1 HPV 16 proteins, has been shown to be safe, highly immunogenic (with anti-HPV IgG titers much higher than those that follow natural infection). It has also proved to be efficacious in preventing persistent HPV infections in a trial of 1523 HPV 16-naive young women in the United States. A multivalent vaccine against the most common oncogenic HPV types may thus ultimately represent the most effective way to prevent cervical cancer world-wide, alone or in combination with screening.

It may, however, take several years before this approach becomes a reality. Therefore, early detection of cervical cancer precursors by screening and their treatment will remain the most important measures for the control of cervical cancer for the foreseeable future.

PROPHYLACTIC CANCER VACCINES

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The immune system effectively limits tumor growth, as illustrated by the enhancement of carcinogenesis, both spontaneous and induced, observed in severely immunodepressed mice. Unfortunately the very existence of tumors, both in mice and in humans, clearly demonstrates that spontaneous immune responses are not sufficient for a complete prevention of carcinogenesis. It has been suggested that a prophylactic activation of the immune system with vaccines and cytokines could result in a significant reduction in tumorigenesis (Cancer Res., 60: 2571, 2000).

The effectiveness of tumor immunoprevention has been clearly demonstrated both for carcinogen-induced tumors and for spontaneous tumors arising in transgenic mice, using a wide array of different immunological strategies based on nonspecific modulators of the immune response like interleukin 12 (IL-12), or on antigen-specific vaccines (Cancer Immunol. Immunother., 51: 409, 2002). An almost complete immunoprevention of mammary carcinogenesis in HER-2/neu transgenic mice was obtained with a cell vaccine combining three different immunologic stimuli, HER-2/neu gene product p185, allogeneic major histocompatibility complex (MHC) class I molecules, and IL-12 (J. Exp. Med., 194: 1195, 2001).

The analysis of protective immune responses revealed some unexpected features. Antibodies and T cell-derived cytokines like γ-interferon played a key role in immunoprevention, at variance with immunotherapy which is firmly based on cell-mediated cytotoxicity. In the HER-2/neu system anti-p185 antibodies, in addition to immunological functions leading to tumor cell lysis, inhibited p185 dimerization and induced its internalization, resulting in the inhibition of mitogenic signaling. A common problem with tumor antigens is the generation of antigen-loss variants that escape immune defenses. In the HER-2/neu model the loss of p185 expression was invariably accompanied by a loss of tumorigenicity, possibly leading to tumor dormancy.

The results suggest that most current tumor antigens are unsuitable targets for cancer immunoprevention (Trends Immunol., 24: 62, 2003). An ideal antigen should have a crucial pathogenetic role in tumor growth to avoid the selection of antigen-loss variants. Downregulation of MHC expression during tumor progression frequently limits antigen recognition by MHC-restricted T cells. Thus an ideal antigen for cancer immunoprevention should be recognized both by T cells and by antibodies. The discovery of new tumor antigens that are directly involved in neoplastic transformation and are recognizable by the immune response also in MHC loss variants will be crucial for the development of cancer immunoprevention and will provide new targets also for cancer immunotherapy.

ADENOVIRUS/DNA VACCINATION AGAINST RAT HER2/NEU IN TRANSGENIC MICE

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The transforming rat HER2/neu oncogene when embedded in the genome of transgenic BALB/c (NeuT) mice provokes the development of an invasive carcinoma in each of their ten mammary glands. We used NeuT mice to evaluate the immunization efficiency and the protective effect of i.m. injection of Adenovirus and/or DNA with electro-stimulation (DNA+ES), both expressing the rat p185 antigen.An optimized rat HER2 cDNA sequence which exclusively contained codons preferred by highly expressed mammalian genes and displayed higher expression in cultured cells and immunogenicity than the non-optimized gene, was used in this study. Adenovirus expressing ratHER2.opt (Ad5-ratHER2.opt) induced a higher immune response when compared to the same gene injected as DNA+ES, as measured by the frequency of IFNγ-secreting spleen cells and antibody titres. Different Ad/DNA combinations and immunization schedules confirmed the superiority of Ad-ratHER2.opt in inducing a strong Th1-skewed humoral and $CD8^+$ cell-mediated response. Two Ad5-ratHER2.opt injections of 10^9 viral particles at week 10 and 12 were sufficient to induce the highest response. An immunization schedule with three Ad injections at week 10, 12 and 19 did not augment the response when compared to the previous protocol. In both cases the response persisted at detectable levels up to week 33. A group which received three injections of DNA+ES at week 23, 27 and 31 in addition to the three Ad injections at week 10, 12 and 19 exhibited a similar response pattern till week 25. Subsequently, however, it showed an increase of the $CD8^+$, $IFN\gamma^+$ PBMC frequency which persisted at detectable levels beyond week 38.

Ad5-ratHER2.opt administration at 10 and 12 weeks of age had a significant impact on tumor progression. At 45 weeks, 60% of the mice were completely protected from tumors and the mean tumor number was < 2.5. In contrast, control mice developed 10 tumors and died by week 27.

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Session VI
Innovative approaches for vaccine therapy and prevention

Chairpersons
G. D'Agnolo, L. Chico Bianchi

PRECISION GUIDING OF CYTOLYTIC T-LYMPHOCYTE RESPONSES

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Molecular triggers of DC activation sufficient for induction of $CD8^+$ CTL responses include agonistic CD40 antibody or ligands of Toll like receptors such as LPS (TLR4 ligand) or CpG (TLR9 ligand). In natural immune responses specific CD4 cells, reactive with peptide antigens presented by MHC class II molecules on DC, can also drive maturation of immature DC to the mature DC state required for $CD8^+$ CTL response induction. $CD4^+$ T helper cells to a large extent operate through upregulation of CD40L which then interacts with CD40 on DC to cause the required DC activation. Important cognate interactions for full $CD8^+$ CTL induction by activated DC are CD80/CD86 on the DC, costimulating CD28 on the CD8 cells. For maintenance and full expansion of CD8+ T cells, interaction of 4-1 BBL (another member of the TNF(R) family) on DC with 4-1 BB on $CD8^+$ CTL is also important. In the absence of CD80/CD86 costimulation, the 4-1 BBL -> 4-1 BB interaction appears to be inactive. Thus proper induction, expansion and maintenance of $CD8^+$ CTL responses involve delicate interactions between $CD4^+$ T-cells, DC and $CD8^+$ T-cells involving several members of the TNF(R) family, including as signal transduction molecules CD40 on DC and 4-1 BB as well as CD27 on $CD8^+$ CTL precursors. Recently we obtained conclusive evidence that immature DC loaded with antigen cause T-cell division but not T-cell effector cell induction, nor T-cell survival in appreciable numbers. LPS stimulated DC, in contrast, stimulated vigorous $CD8^+$ CTL responses *in vivo*. Such $CD8^+$ effector cells showed loss of CD62L and CCR7 lymphoid homing receptors, compatible with their migration into blood and parenchymal tissue in large numbers.

We recently investigated the conditions for optimal therapeutic $CD8^+$ CTL induction by long peptide vaccins against human papillomavirus induced mouse tumors. The 32-35 amino acid long peptides were given SC in IFA or in CpG 1826 adjuvant. Powerful therapeutic CTL induction by single peptide vaccination crucially depends on coinjection at the same site of CpG adjuvant and this response was MHC class II independent. In prime-boost regimes a second mechanism started contributing to CTL induction, namely $CD4^+$ T helper cell mediated CD40L dependent activation of DC. Toll like receptor triggering is therefore very useful in $CD8^+$ CTL priming, while CD40L activation starts operating in boosting.

In addition, quite apart from their activation of $CD4^+$ helper cells, long peptides are superior to exact MHC class I binding peptides. It appears that the exact MHCI binding peptides indiscriminately bind to all MHCI positive cells, including B-cells and T-cells. The latter cell types, loaded with exact MHC binding peptides, recirculate and tolerize the immune system. Long peptides, in contrast, need to be processed by professional APC. As

a result only professional APC present MHC I and II epitopes processed from long peptides *in vivo*.

The combined data show that a new powerful generation of therapeutic anti-cancer vaccines consists of completely synthetic compounds: specific long synthetic peptides with or without molecularly defined adjuvants.

HOW LYMPHODEPLETION ENHANCES AUTOIMMUNITY AND CANCER REGRESSION UPON ADOPTIVE TRANSFER OF T LYMPHOCYTES

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Immunotherapy utilizing expanded tumor-infiltrating lymphocytes following administration of a non-myeloablative but lymphocyte-depleting regimen has been successfully employed for the treatment of patients with advanced cancer. However, the biological mechanisms by which a non-myeloablative regimen may augment immunotherapy therapy have not been clearly elucidated. To test if non-myeloablative conditioning could enhance cancer regression in a mouse model using large, established tumors, we employed the pmel-1 murine model for the treatment of B16 melanoma. We utilized the adoptive transfer of tumor/self reactive $CD8^+$ T cells against an epitope derived from a self/tumor antigen, gp100. We show that local irradiation of 500 or 1000 rads had minimal impact on the highly aggressive B16 melanoma and that there were comparable numbers of pmel-1 T cells noted in wild-type vs. non-myeloablative conditioned groups. However, pmel T cells removed from the non-myeloablative conditioned hosts compared to WT non-irradiated controls were functionally enhanced based on IFN-γ secretion. This functional improvement appeared to be due to multiple factors, including: lack of $CD4^+CD25^+$ T regulatory cells and better access to supportive cytokines. We found that the effects of non-myeloablative radiation could be mimicked in syngeneic mice made lymphopenic by genetic modification. SCID, RAG-1 KO, and CD4 KO mice had a remarkable improvement in tumor regression comparable to irradiated wild-type C57BL/6 controls. However CD8 KO was only comparable to non-irradiated wild-type control mice. In add back experiments into RAG-1 KO tumor-bearing hosts, $CD4^+CD25^-$ T cells augmented immunotherapy in the absence of exogenous IL-2, but whole, unfractionated $CD4^+$ T cells or $CD4^+CD25^-$ T cells from IL-2 KO mice did not. In addition, non-myeloablative conditioning of RAG-1 KO or CD4 KO, which do not have $CD4^+CD25^+$ regulatory T cells, further augmented the antitumor responses and autoimmunity, but not in CD8 KO mice. Treatment was impaired when wild-type splenocytes were added back to ablated recipients; treatment was also impaired in ablated IL-15 KO but not wild-type IL-15 KO hosts. Moreover, therapy could be improved by adding supportive exogenous cytokines to ablated recipients. Remarkably, the effects of adding exogenous IL-2, IL-7, and IL-15 appeared synergistic. Collectively, these data demonstrated that lymphodepletion enhances autoimmunity and cancer regression by eliminating $CD4^+CD25^+$ T_{reg} cells and by improving access of adoptively transferred T cells to activating cytokines.

FIRST PHASE I CLINICAL TRIAL USING DENDRITIC CELL DERIVED-EXOSOMES : NK CELL ACTIVATION AS A SURROGATE MARKER OF EXOSOME BIOACTIVITY AND CLINICAL EFFICACY

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Purpose: We described dendritic cell (DC) derived-exosomes as nanomeric vesicles harboring functional MHC molecules capable of promoting T cell immune responses associated with mouse tumor rejection. Here we report the feasability and safety of the first Phase I clinical trial using autologous exosomes pulsed with MAGE 3 peptides purified according to good manufactoring processes (GMP) for the immunization of stage III/IV melanoma patients. Secondary endpoints were the monitoring of $CD4^+$ and $CD8^+$ T cell responses specific for the immunizing MAGE3 peptides and clinical outcome.

Patients and methods: Exosomes were harvested from autologous monocyte derived-DC cultures according to good manufactoring processes. Fifteen patients bearing melanoma fullfilling the inclusion criteria (stage IIIB and IV, HLA-$A1^+$, or -$B35^+$ and HLA-$DPO4^+$ leukocyte phenotype, tumor expressing MAGE-3 antigen) received four exosome vaccinations intradermally and subcutaneously at 1 week intervals. Two dose levels of either MHC class II molecules (0.13 versus $0.40x10^{14}$ molecules) or peptides (10 versus 100 μg/ml) were tested. Patients were clinically evaluated two weeks after the last vaccination. Immunomonitoring of T and NK cell responses were performed using Elispot assays and cytotoxicity, IFNγ Elisa respectively before and following immunization with exosomes.

Results: The GMP process allowed to harvest about 5.4×10^{14} (range: 1.2-16.0) exosomal MHC class II molecules from autologous DC cultures allowing inclusion for the 15 patients. There was no grade II toxicity and the maximal tolerated dose was not achieved using $0.4x10^{14}$ MHC class II molecules pulsed with 100μg of peptides. Five out of fifteen patients exhibited clinical responses (1 partial, 1 minor, 2 stable, 1 mixed) mostly interesting skin and lymph node sites. Exosome-based immunization could not significantly boost MHC class I or II-restricted T cell responses. However, exosome inoculation could promote expansion of circulating $CD3^-/CD56^+$ NK cells in all patients and enhanced NK cell effector functions in 8/13 patients, leading to autologous tumor recognition (3/3 cases) and tumor invasion (in one evaluable tumor). In 3/5 patients exhibiting tumor regression, NK cell effector functions were boosted by vaccination with exosomes. This clinical trial

suggested that GMP exosomes could directly activate NK cells which was confirmed by ex vivo assays.

Conclusion : The first exosome Phase I trial highlighted i) the feasability to purify DC derived-exosomes from metastatic melanoma patients allowing up to 40 vaccines from one leukapheresis , ii) the safety and bioactivity of exosomes at both dosages of MHC class II exosomal molecules and peptides, iii) NK cell activation as a surrogate biological and clinical marker of vaccination with exosomes.

BYSTANDER SUPPLY OF CYTOKINE AND CO-STIMULATION IN CANCER VACCINE

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Transduction of genes encoding cytokines or costimulatory molecules can greatly improve the immunogenicity of tumor cells intended for vaccine use. Complex technical and practical obstacles concerning the choice of vector or the use of autologous versus allogeneic tumor cells hamper translation of this simple approach to the clinical setting. A fully autologous approach may be limited by the availability of tumor cell lines and by the need to genetically modify tumor cells from every patient, a costly and labor-intensive task. A possible alternative is the use of autologous tumor cells mixed with genetically modified bystander cells, which can be fibroblast- or tumor-derived. Although human fibroblasts obtained from cancer patients have been used in clinical trials a universal gene-modified bystander tumor cell line admixed with autologous tumor cells is likely the easiest approach in patients.

Class I-negative B78H1 cells transduced to express IL-12 and mixed with autologous A20 tumor cells led to eradication of pre-established A20 lymphoma in 50% or 100% of treated mice after 3 or 4 vaccinations, respectively, whereas A20 cells alone or mixed with non-transduced B78H1 cured none or 50% of mice after 3 or 4 vaccinations, respectively. Immunization with the IL-12-producing bystander cell line increased tumor-specific proliferation and type 1 cytokine production by $CD4^+$ T cells. By contrast, CD4 T-cell function appeared impaired after immunization with A20 cells alone or mixed with B78H1 cells. Indeed, only $CD4^+$ T cells from IL-12-treated mice could be restimulated with anti-OX40R mAb in place of a fourth cellular boost.

In a different model we tested the effect of GM-CSF on DC and of OX40L on T cells by combining their expression in a cancer cell line. C26 cells transduced with both GM-CSF and OX40L were rejected by 90% of the injected mice and when used irradiated as cellular vaccine cured 85% of mice with lung metastases. Of interest while a mixture of live C26 cells transduced with either GM-CSF or OX40L was not rejected, the same mixture, irradiated, showed therapeutic activity against lung metastases. Foreseeing a bystander approach, we are testing whether GM-CSF can be provided at priming phase while OX-40 at boosting phase, and whether bystander cells producing GM-CSF and OX40 agonistic Ab can substitute the syngeneic cells in vaccine formulation.

MODULATION OF TUMOR ANTIGEN EXPRESSION TO IMPROVE CANCER VACCINE-BASED IMMUNOTHERAPY

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Antigenic heterogeneity is a characteristic shared by most, if not all, tumor cell populations. Moreover, down-regulation of major histocompatibility (MHC) antigens has been proposed as one possible pathways by which tumor cells escape immune recognition. Studies have been carried out that identify specific agents, such as cytokines and chemotherapeutic drugs, which can modulate the expression of tumor-associated as well as MHC antigens on the tumor cell surface. Two of the most commonly studied agents, the interferons (IFN) and 5-fluorouracil (5-FU), have been shown in experimental as well as clinical studies to upregulate tumor and normal MHC antigen expression. Cellular and molecular studies have established that both interferon-alpha and –gamma can significantly increase MHC-specific mRNA levels, resulting in enhanced expression of those antigens on the tumor cell surface, as confirmed by flow cytometry. In a recent study, IFN-gamma treatment of murine colorectal tumors *in vivo* significantly increased their susceptibility to T-cell-mediated cytolysis. Likewise, preliminary experimental results have implicated the use of 5-FU and other chemotherapeutic compounds to increase tumor cell killing through modulation of the Fas-mediated cytolytic pathways. Those results underscore the need to identify agents that can alter the susceptibility of tumor cells to cell-mediated killing. These results will be a crucial addition to the ongoing development of therapeutic-based cancer vaccines.

ANTIBODY MEDIATED TUMOR TARGETING OF ANTIGENIC MHC/PEPTIDE COMPLEXES AS A NEW FORM OF CANCER THERAPY, FIRST ENTIRELY *IN VIVO* RESULTS

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The new cancer immunotherapy strategy described here consists in using Fab' fragments from a high affinity anti-TAA mAb coupled to a MHC class I, containing a selected antigenic peptide of non tumor origin, in order to target the active MHC/peptide complex on tumor cells and induce their lysis by specific CTLs. To demonstrate the *in vivo* antitumor efficacy of this approach, we took advantage of OT-1 C57BL/6 mice, in which most CD8 T lymphocytes express a transgenic TCR specific for the ovalbumin immunodominant peptide (257-264) in the context of H-2Kb. First, we subcutaneously grafted a CEA-transfected syngeneic colon carcinoma cell line (MC38-CEA^{+}) in OT-1 mice and immediately treated them by serial injections of an anti-CEA-H-2Kb /ova conjugate. Control mice were injected similarly but only with the antibody part as F(ab')2 fragment. The results at day 28 showed a highly significant difference of tumor size between the two groups, with a mean tumor volume of 77 mm^3 for the conjugate treated mice and 633 mm^3 for the control mice. This experiment was repeated with essentially similar results. In a second setting, spleen cells from OT-1 mice were adoptively transferred in CEA transgenic mice, followed by immunization with ovalbumin. Animals were then subcutaneously grafted with the same syngeneic colon carcinoma line and at day 8, when all the tumors were palpable, mice were treated by systemic injections of the anti-CEA-H-2Kb /ova peptide conjugate or only anti-CEA F(ab')2. At day 24, four out of five mice from the conjugate treated group had tumor volumes of less than 20 mm3, while all mice from the control F(ab')2 group had tumors bigger than 200 mm3, as did one of the conjugate treated mice, which escaped tumor treatment.

These first entirely *in vivo* results of antibody-mediated targeting of MHC complexes containing an antigen of non tumor origin are very encouraging and we are currently testing this strategy in a new *in vivo* model with LCMV anti-viral CTLs targeted to B16 melanoma-induced lung metastasis. Potential clinical application of this immunotherapy strategy would exploit the T cell memories that any individuals have developped against various viral infections, such as EBV, CMV or Influenza and redirect them against cancer cells. Besides exploiting strong viral antigens as compared to usually low affinity tumor T cell epitopes, this approach would also hinder tumor escape that often occurs by down modulation or loss of MHC Class I expression by the tumor cells.

DENDRITIC CELLS GENERATED WITH TYPE I IFN: POTENTIAL ADVANTAGES FOR THEIR USE IN THE DEVELOPMENT OF THERAPEUTIC VACCINES

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Dendritic cells (DC) are the most promising cellular adjuvants for the development of cancer vaccines. However, there are still uncertainties on the mechanisms controlling differentiation/activation of DC as well as on the types of DC exhibiting optimal characteristics for clinical use. Recently, evidence obtained in mouse and human models have indicated type I IFN as important factors for DC differentiation/activation. Of note, studies on human GM-CSF-treated monocytes have suggested the potential advantage of using IFN for the preparation of active DC for clinical studies. In fact, IFN-α induced the rapid generation of DC (IFN-DC) endowed with potent migratory and Th-1 priming immune activities *in vitro* as well as in hu-PBL-SCID mice vaccinated with autologous antigen-pulsed DC (J. Exp. Med. 191:1777, 2000; Blood 98:3022, 2001). Notably, IFN-DC were highly efficient in inducing a cytotoxic $CD8^+$ T cell response against EBV antigens (J. Immunol. 170:5195, 2003) and against HIV-1 antigens in hu-PBL-SCID mice (J. Exp. Med.198:361, 2003). IFN-DC were superior with respect to immature DC, generated after treatment of monocytes with GM-CSF and IL-4 (IL-4-DC), in inducing a protective immune response. Of interest, IFN-DC generated from monocytes of patients with chronic myelogeneous leukemia were highly active in inducing the generation of autologous $CD8^+$ T cells reactive against leukemic cells (Blood 103:980, 2004). Our recent data indicate that IFN-DC, despite their partially mature phenotype, maintain a phagocytic activity similar to that exhibited by immature DC. Interestingly, the expression levels of TAP molecules and of the catalytic subunits of the immunoproteasome were found to be similarly up-regulated in IFN-DC and in fully mature DC with respect to immature DC. We recently assessed the gene expression profiles in IFN-DC compared to IL-4-DC, and found that a 3-day IFN-α treatment of human monocytes induced the up-regulation of 54 significant genes and the down-regulation of 73 genes. In addition to a strong induction of the IFN-inducible genes (2'-5'-oligoadenylate synthetase, Rnase L, pkR) and of transcription factor genes of the IRF family, we observed a considerable up-regulation of a number of cytokine, chemokine and chemokine receptor genes. These results indicate that IFN-DC are characterized by a gene expression profile associated with the acquisition of features typical of mature highly active DC. On the basis of the preclinical data showing the powerful activity of IFN-DC, we are currently attempting to develop GMP procedures for the preparation of IFN-DC suitable for clinical applications.

Session VII
Dendritic cells and clinical trials

Chairpersons
F. Cognetti, S. Pecorelli

DENDRITIC CELL BASED VACCINES IN MOUSE AND MAN

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Dendritic cells (DC) have the unique capacity to attract, interact and activate naive T cells to initiate immune responses. The ability of DC to initiate immune responses allows their exploitation in therapeutic strategies against cancer and other diseases. DC based vaccinations strategies are studied, developed and optimized in mouse tumor models. We have also performed clinical studies in which we evaluated the capacity of mature and immature human monocyte derived DC loaded with KLH protein and gp100 and tyrosinase peptides to migrate and stimulate immune responses in melanoma patients. The immunological and clinical data of the first 20 patients entered in the trials indicate that mature DC are far superior to immature DC to induce immune responses *in vivo*.

To further optimize clinical efficacy of DC-based vaccines in cancer patients, proper monitoring of the immune response is of utmost importance. We have now investigated the possibility to monitor responses using skin biopsies derived from a delayed type hypersensitivity (DTH) sites after injection with unloaded or peptides and/or KLH loaded DC. Short term culture of the biopsies demonstrated the presence of MHC tetramer positive T cells, but only when the DTH was performed with peptide-loaded DC. Cell cultures containing tetramer-positive T cells also exhibited functional activities. Furthermore, MHC-tetramer positive cells were detected by immuno-fluorescent staining on sections of the biopsy, demonstrating that peptide-specific T cells were already present in situ. The presence of antigen-specific T cells in the DTH of patients doing clinically well, suggests that the evaluation of T cell reactivity in positive DTH sites may be a powerful tool in the monitoring of clinical T cell-directed vaccination studies in cancer patients.

DENDRITIC CELL-BASED IMMUNOTHERAPY FOR GYNECOLOGIC CANCERS

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The recent recognition of dendritic cells (DC) as powerful antigen-presenting cells capable of inducing primary T cell responses *in vitro* and *in vivo*, in combination with the identification of tumor-specific/associated antigens, has generated widespread interest in DC-based immunotherapy against gynecologic tumors. In ovarian cancer, the cloning and sequencing of CA125 as well as of several novel ovarian tumor antigens, including hepsin, stratum corneum chymotryptic enzyme (SCCE), and the tumor-associated differentially expressed gene (TADG)-12, TADG-14, TADG-15 and TADG-16, all of which are serine proteases, have recently been described. These proteins have been proposed as attractive candidates as novel tumor antigens for ovarian cancer immunotherapy. Consistent with this view, our laboratory has recently used two computer algorithms to identify candidate peptide sequences within TADG-12 and CA125 that have the potential to bind to HLA class I molecules. Cytotoxic T lymphocyte (CTL) epitopes against TADG-12 and CA125 have been identified and some of these HLA-A2-binding peptides have been shown to elicit potent tumor specific HLA-class I-restricted cytotoxicity against autologous ovarian tumor cells. These results will be instrumental for the design of an effective DC-based vaccination strategy against TADG-12 and CA125-positive ovarian tumors *in vivo*.

Abundant evidence indicates that human papillomavirus (HPV), predominantly of the HPV 16 and 18 genotypes, plays an important etiologic role in the development of cervical cancer. Our group has provided *in vitro* evidence that HPV E7 antigen-loaded DC can efficiently induce both E7-specific helper $CD4^+$ T cell responses and E7-specific $CD8^+$ CTL that are capable of killing autologous tumor cells from cervical cancer patients. More recently, we have initiated clinical trials in women with advanced and/or recurrent HPV16 and/or HPV18 cervical cancer. Although the majority of cervical cancer patients have been found to be severely immunocompromised at the time of DC-vaccination, preliminary data suggest that E7-pulsed autologous DC vaccination holds potential as a novel adjuvant treatment for patients harboring recurrent and/or metastatic HPV-positive cervical cancer.

VACCINATION WITH MONOCYTE-DERIVED DENDRITIC CELLS: "LEARNING BY DOING"

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Most of the dendritic cell (DC) vaccination studies have been performed by using DC generated from $CD14^+$ monocytes (so-called Mo-DC), but often immature DC or DC matured in the absence of PGE2 were used, which might result in tolerance induction or non-migratory Mo-DC, respectively. We have performed a series of DC vaccination trials accompanied by serial immunomonitoring in metastasizing melanoma patients after extensive preclinical validation. Our DC vaccine consists of tumor-peptide loaded Mo-DC matured initially by autologous monocyte-conditioned medium and later by its mimick (i.e. IL-1 beta + IL-6 + TNF alpha + PGE2). In initial trials (19 patients) we vaccinated with Mage-3 peptide-loaded DC and demonstrated the induction of Mage-3 specific CTL responses which have proven polyclonal. We then optimized the cryopreservation of DC which allowed us to use in the next "multi-peptide" trial (16 patients) frozen aliquots of DC which were loaded upon thawing with MHC class I and II restricted peptides (peptides were loaded so that no competition for a particular HLA molecule occurred, and 4 million DC per HLA-A1,-A2.1, and -A3 restriced peptide were used). We observed a rapid induction of Th1 cells. In contrast, the induction of CTL was weak except in select patients. In a still ongoing follow-up trial (about 40 patients) we are now using ready to use peptide-loaded and cryopreserved DC which in a second cohort are injected even without removal of the cryoprotectant DMSO with comparable results. In this trial the number of DC carrying a given class I peptide was increased from 4 to 10 million DC which resulted in a reproducible priming or expansion of tumor-peptide specific $CD8^+$ T cells. In our trials we have observed in a significant proportion of patients stabilization of disease and regression of part of the metastases. Mean overall survival increased from 11-15 months in previous trials to 25^+ months in the closed "multipeptide trial", and will presumably be higher in the still ongoing trial. Following extensive preclinical work we have now started to vaccinate stage IV melanoma patients with DC transfected with RNA coding for MelanA, Mage-3 and Survivin.

MELANOMA THERAPEUTIC VACCINE BASED ON DENDRITIC CELLS LOADED WITH ALLOGENEIC TUMOR CELL LYSATES AS ANTIGEN SOURCE

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Dendritic cell (DC) based vaccines are a promising cancer therapeutic approach. In addition, allogeneic lysates from tumor cell lines represent an attractive source of antigens to load on DC: They contain multiple known and unknown tumor-associated antigens (TAA), could potentially trigger both CD8 and CD4 T cell response, and their production can be standardized for a clinically compatible process. A phase I trial recently completed demonstrated the safety of this DC/lysate combination in 15 melanoma patients treated with DC pulsed with an allogeneic melanoma lysate. No grade 3 or 4 adverse events related to the vaccination were observed. Specific immune responses to the lysate and tumor associated antigens were detected after vaccination. Two out of 8 evaluable patients showed signs of clinical response after 4 vaccinations. One patient showed regression of in-transit metastases leading to complete remission for >19 months and a second patient remained stable for >4 months.

We have also developed a production process including maturation agents of clinical grade: DC/lysate exposed to these maturation agents before cryopreservation were committed to maturation, as determined by their expression of specific surface markers and the secretion of high levels of IL-12p70.

Based on these results, a randomized phase I/II clinical trial has been initiated in stage IV melanoma patients to compare non-matured or matured DC/lysate vaccine.

Session VIII
Tracking the anti-tumor immune response

Chairpersons
E. Bonmassar, G. Francini

TUMOR MICROENVIRONMENT AND IMMUNE RESPONSES TO TUMOR ANTIGEN-SPECIFIC IMMUNIZATION

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The recent progress in tumor immunology exemplifies the successful application of modern biotechnology for the understanding of the complex natural or therapy-induced phenomenon of immune-mediated rejection of cancer. Tumor antigens recognized by T cells were identified and successfully utilized in active immunization trials for the induction of tumor-antigen specific T cells. This achievement has left, however, the clinicians and researchers perplexed by the paradoxical observation of the immunization-induced T cells can recognize tumor cells in standard assays but most often cannot induce tumor regression. In this presentation, we will argue that 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. We believe that the key to the understanding of this complex phenomenon relies on the real-time study of tumor/host interactions in the tumor microenvironment. Most likely, T cells induced by immunization can reach the tumor site but they are not capable of performing their effector function because they encounter a tumor microenvironment not conducive to T cell activation. We recently characterized a quiescent cytotoxic T cell phenotype that can respond to antigen stimulation by secreting cytokines but cannot kill target cells nor proliferate unless a secondary stimulation is provided such as interleukin-2. This phenotype is characteristic of immunization induced T cells and may explain the discrepancy between their observation in the circulation and the lack of tumor regression.

QUANTITATIVE AND QUALITATIVE ASSESSMENT OF PEPTIDE VACCINE INDUCED CD8 T CELL RESPONSES IN MELANOMA

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Tumor antigen-specific T cells, similar to other types of antigens, occur at relatively low frequencies. Thus, direct measurement of the numbers as well as the functional properties of such cells remains challenging.

Major progress in this direction has been made in recent years. Today, multicolor flow cytometry based analysis allows the enumeration and phenotypic cataloguing of antigen-specific T cells. A useful combination of markers is the use of MHC class I/peptide multimers in conjunction with the CD45RA, CCR7, CD27 and CD28 cell surface markers. CD8 T cell functions can also be directly evaluated with flow cytometry based assays. These include cytokine production recorded at the single cell level, expression of effector molecules such as granzyme B and perforin and monitoring of granule exocytosis using as indicator the transient appearance of the LAMP-1&2 proteins at the cell surface.

A critical parameter of the anti-tumor efficacy of cytolytic T lymphocytes is the avidity of antigen recognition. In turn, a major determinant for functional avidity is the intrinsic affinity of the T cell receptor for antigen (TCR). While there are no direct techniques available to measure TCR avidity, mutated class I MHC molecules with highly reduced CD8 binding due to substitutions in a conserved, negatively charged loop of the heavy chain $\alpha 3$ domain, have been exploited to visualize high avidity tumor-reactive T cells. Such CD8-null multimers provide a very useful tool for the ex vivo comparison of vaccination strategies for their ability to enhance the frequency of high avidity CTL. They also enable their selective isolation for adoptive transfer therapy.

It is now possible to accurately track the evolution of single antigen-specific T cells directly ex vivo. Thus, careful clinical trial design with immunlogical end points should lead to rapid progress in the learning process of efficient therapeutic vaccination. I will illustrate these issues with recent results of monitoring phase I clinical trials of peptide based cancer vaccines in metastatic melanoma.

T CELL DIFFERENTIATION IN MELANOMA PATIENTS: A NEW TOOL IN THE ANALYSIS OF T CELL-MEDIATED IMMUNITY TO TUMOR ANTIGENS

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Current models of antigen-induced $CD8^+$ T cell differentiation define T cell maturation as a linear sequence. $CCR7^+$ $CD45RA^+$ naive (T_N) cells differentiate first to Ag-experienced $CCR7^+$ $CD45RA^-$ central memory (T_{CM}), and $CCR7^-$ $CD45RA^-$ effector memory (T_{EM}) cells, and then to $CCR7^-$ $CD45RA^+$ terminally differentiated (T_{TD}) stage. Each step in this process is seen as part of a continuum, and some of the stages can be reversible. Nevertheless, by applying these models it is possible to dissect the evolution of anti-tumor immunity in cancer patients. In this study, we evaluated T cell differentiation at tumor site in a large panel of tumor-invaded lymph nodes (TILN) from Stage III melanoma patients. T cell differentiation was compared in these patients to the phenotype of T lymphocytes isolated from tumor-free lymph nodes (TFLN) removed from the same nodal basins as the TILN. In all these tissue samples, we investigated $CD8^+$ T cell phenotype by evaluating expression of CCR7, CD45RA, and cytolytic factors. By hierarchical cluster analysis, we identified four different maturation clusters. In the first, more immature cluster, $CD8^+$ T cells from all TFLN (n=42) and from 56% of the TILN (n=142) were grouped. This cluster was mainly characterized by T_N or T_{CM} cells lacking granzyme B or perforin. Three additional clusters contained only T cells from the remaining TILN and showed a progressive increase in the content of T cells at the T_{EM} or T_{TD} stage with expression of cytolytic factors. These different maturation clusters, found at the level of the bulk T cell population, were confirmed by analysis of the phenotype of antigen-specific T cells, isolated from the same tissue samples, and identified by HLA tetramers specific for several melanoma-associated antigens. Moreover, the maturation phenotype of $tetramer^+$ T cells could predict the functional status of these cells, as lymphocytes at the T_{EM} stage showed an enhanced outgrowth (compared to T_N cells) upon stimulation with the cognate antigen presented as a peptide by autologous antigen-presenting cells. Moreover, $tetramer^+$ T cells at the T_{EM}, but not at the T_N stage, could release IFN-γ in response to the cognate antigen, without any pre-activation, although functional maturation of T_N cells could be achieved by culture with IL-2 or IL-15. Taken together, these results indicate that $CD8^+$ T cells at the $CCR7^-$ cytotoxic $factor^+$ stages are present in TILN, but not in TFLN, of a relevant fraction of melanoma patients and suggest that cytokines such as IL-2 and IL-15 may be exploited to promote Ag-independent maturation of anti-tumor $CD8^+$ T cells.

ANALYSIS OF T CELL PERSISTENCE IN MELANOMA PATIENTS RECEIVING ADOPTIVE IMMUNOTHERAPY

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Objective clinical responses have been observed in approximately 50% of melanoma patients that were treated with a non-myeloablative chemotherapy protocol prior to the adoptive transfer of polyclonal populations of *in vitro* cultured tumor infiltrating lymphocytes (TIL). In a previous report, individual clones of tumor reactive T cells derived from TIL represented >70% of the circulating peripheral blood lymphocytes in 2 patients for a period of over 4 months following transfer, during which time the nearly complete regression of multiple metastatic lesions was observed. The current studies were undertaken to examine the relationship between *in vivo* T cell persistence and the clinical responses of additional patients that were treated as a part of this trial, as well as to evaluate factors that are involved with maintaining T cell persistence. The degree of persistence of individual T cells clones was evaluated by analyzing T cell receptor beta chain variable region (TR-BV) expression by FACS and by sequencing the TR-BV gene products expressed in TIL and samples of peripheral blood lymphocytes (PBL) obtained at different times following adoptive transfer. Analysis of the expressed TR-BV sequences, which represent essentially clonal markers, was carried out using a 5' RACE technique that provides a quantitative measure of the representation of individual T cell clones in polyclonal populations of cells.

The results demonstrated that individual T cell clones present in polyclonal TIL varied widely in their ability to persist *in vivo* following adoptive transfer. Tumor reactive T cell clones that were present at similar or higher relative levels in PBL than in the adoptively transferred TIL were identified in several patients, while in the same patients additional clones of tumor reactive T cells demonstrated a dramatic decline in their relative levels following adoptive transfer. Analysis of the phenotype of persistent T cells demonstrated that these cells expressed high levels of the co-stimulatory markers CD28 and CD27 as well as the alpha chain of the IL-7 cytokine receptor, and current studies are being carried out to determine the impact of the expression of these markers on the persistence of adoptively transferred T cells.

THE USE OF MICRO ARRAY TECHNOLOGY FOR THE MOLECULAR TRACKING OF ANTI-TUMOR IMMUNE RESPONSES

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Anti-cancer immune responses are a natural phenomenon that can be enhanced by immune manipulation. The biological mechanism responsible for this phenomenon remains largely unexplained. Conventional immunology has extensively studied specific interactions between immune and cancer cells. Additional investigations have identified co-factors that may enhance the effectiveness of such interactions. As the molecular understanding of individual interactions increases, it is becoming apparent that no single mechanism can in itself explain the phenomenon of tumor rejection. Most likely, the contribution of several components of the innate and adaptive immune response is required for successful tumor rejection. These components may be variably recruited and activated within the tumor microenvironment by the production of molecules with immune modulatory properties by tumor and bystander cells. Such complexity can only be appreciated and solved by high throughput tools capable of providing a global view of biological processes as they occur.

We have previously suggested that 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 we observed 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. We are presently evaluating various mechanisms responsible for the activation of immune responses at tumor site using genomic as well as proteomic based approaches.

Poster abstracts

1 EPIDERMAL GROWTH FACTOR (EGF) BASED CANCER VACCINE FOR NON SMALL CELL LUNG CANCER (NSCLC) THERAPY: ANALYSIS OF POOLED DATA FROM THREE CLINICAL TRIALS

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Epidermal Growth Factor promotes cellular proliferation and survival upon binding to its receptor (EGF-R) in tumors of ephitelial origin. During the last 10 years we have studied the effect of vaccination against self EGF, both in the preclinical setting, and in several pilot clinical trials in advanced NSCLC patients. Here, we undertake the analysis of aggregated data from these trials, addressing particularly the issue of the relationship between immunization and survival.

Pooled data from 3 pilot clinical trials in 75 patients, considered not amenable to any other modality of onco-specific treatment, were used. For survival analysis, 27 NSCLC patients were considered as a non-randomized concurrent control group. This group of patients has features which make it adequate for comparison: compliance with inclusion criteria, simultaneity in time, and treatment in the same hospital by the same physician staff. Vaccination using different adjuvants (alum and Montanide ISA51), cyclophosphamide pre-treatment or not, as well as different dosages of the Vaccine were compared.

Eighty percent of vaccinated patients showed seroconversion, of which 47% developed a good antibody (Ab) response. Geometric mean of maximal Ab titers of patients with seroconversion was 1:3949 (sera dilution). Adjuvant, vaccine dose and cyclophosphamide pre-treatment significantly influenced immunogenicity whereas the other analyzed variables (sex, age, clinical stage, previous treatment) did not. The immune response was short lasting (2.64 +/- 1.89 months) and non-boostable. To maintain Ab titers continuous re-immunizations were required. No serious adverse events were registered. Vaccinated patients survived significantly more (mean SV: 9.13 months; median SV: 8 months) than the control group (mean SV: 4.85 months; median SV: 4.53 months) ($p=0.0003$). Patients who seroconverted survived significantly more than patients who did not. Patients with good Ab response showed a significant improvement in survival as compared with those with poor Ab response.

Conclusions: Vaccination with EGF was safe, immunogenic and improved survival in advanced NSCLC patients as compared with concurrent controls. These results are currently being verified in a randomized trial.

2 XENOGENEIC IMMUNIZATION IN MICE USING HER2/NEU DNA DELIVERED BY AN ADENOVIRAL VECTOR

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The protective efficacy of xenogeneic vaccination with DNA encoding the HER2/neu oncogene was evaluated in BALB/c mice transgenic for the transforming form of the rat HER2/neu oncogene which spontaneously develop carcinomas in all mammary glands. Intramuscular injection of either plasmid DNA followed by electrical stimulation (pVij-HER2 with ES) or an Adenoviral vector (Ad5-HER2), both expressing the HER2/neu oncogene, were tested. Immunization using pVij-HER2 with ES elicited a cell-mediated response that was much lower than that elicited by the immunization with Ad5-HER2, as measured by the frequency of IFN-γ secreting spleen cells. The dominant T-cell epitope of the HER2/neu protein product (p185) in the BALB/c (H-2^d) genetic background was identified. While the T cell response elicited was only partially cross-reactive with the corresponding rat epitopes because of sequence variations (89% similarity), a cytotoxic T lymphocyte activity against the rat immunodominant epitope was also evident. The Ad5-HER2 vaccination induced also antibodies against p185 which cross-reacted with the rat protein homologue. Both T- and B-cell responses slowly declined with time. Vaccination with Ad5-HER2 at 6 and 9 weeks of age delayed tumour incidence and reduced tumour multiplicity in rat HER2/neu transgenic mice.

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3 TOWARDS INDUSTRIALIZATION OF A MELANOMA THERAPEUTIC VACCINE BASED ON DENDRITIC CELLS LOADED WITH ALLOGENEIC TUMOR CELL LYSATES AS ANTIGEN SOURCE

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We have standardized a clinically compatible process to generate large quantities of monocyte-derived dendritic cells (DCs), in serum-free medium containing GM-CSF and IL-13. Allogeneic lysates from tumor cell lines are an attractive source of antigens: they contain multiple known and unknown tumor associated antigens (TAA). We have previously shown that DCs loaded with melanoma lysate cross-prime CD8 T cells specific for TAA *in vitro*, and developed a clinical grade production process of a melanoma vaccine based on lysate-loaded matured DCs (Uvidem).

In order to industrialize the production of Uvidem, we developed a procedure to further automate the process and allow release of cryopreserved doses. The wash of apheresis products from healthy donors, and transferring them into culture bags afterwards, were automated using the CytoMate device. After culture and purification by elutriation, DCs (n = 3) had viability higher than 95% and expressed surface molecules typical of immature DCs. After overnight loading with melanoma lysates and maturation for 6 hours with a clinical grade bacterial extract in combination with IFN-gamma, DCs were washed with the CytoMate to eliminate process residuals, and further resuspended in cryopreservation medium. This process allowed the recovery of an average of 50% of DCs, with viability and purity higher than 80%. In addition, DCs were committed to maturation, as shown by increased expression of HLA and costimulatory molecules, expression of CD83, and secretion of IL-12p70 and TNF-alpha after overnight culture. Testing after cryopreservation in liquid nitrogen showed that on average, 70% of cryopreserved DCs were recovered. Importantly, the freezing and thawing steps did not alter viability, purity, surface markers, and cytokine secretion. Moreover, thawed DCs could be kept for at least 1 hour at room temperature before injection.

Using this process, phase II trials will be initiated to assess the clinical activity of Uvidem.

4 LOW DOSE IRRADIATION OF HUMAN TUMOR CELLS MODULATES PHENOTYPE RESULTING IN ENHANCED KILLING BY CTL

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Local radiation of tumor masses is an established modality for the therapy and/or palliation of a range of human tumors. Radiation has also been shown to be capable of altering the phenotype of target tissue, including gene products that may make tumor cells more susceptible to T-cell–mediated immune attack. Previously, we demonstrated that radiation increased Fas (CD95) gene expression in murine MC38-CEA$^+$ tumor cells, which, consequently, enhanced their susceptibility to CEA specific CTL mediated killing. The present study was designed to extend these observations to human tumor cells. Here, 24 human tumor cell lines (12 colon, 7 lung, and 5 prostate) were examined for their response to non-lytic, low dose radiation (10 or 20Gy). Seventy-two hours post-irradiation changes in surface CD54 (ICAM-1), CD95 (Fas), CD227 (MUC-1), CD66 (CEA) and class I, expression were examined. Eighteen, of the 24, cell lines upregulated one or more of these surface molecules. Furthermore, five of five irradiated CEA$^+$/A2$^+$ colon tumor cells lines were killed better by CEA specific HLA-A2 restricted CD8$^+$ CTL than their non-irradiated counterparts. Finally, we utilized microarray analysis to broaden the scope of observed changes in gene expression following radiation, and found that many additional genes had been modulated. These upregulated gene products could further enhance the tumor cells susceptibility to T-cell–mediated immune attack as well as serve as additional targets for immunotherapy. Overall, the results of this study suggest that non-lethal doses of radiation can be used to make human tumors more amenable to immune system recognition and attack and form the rational basis for the combinatorial use of cancer vaccines and local tumor irradiation.

5 DRUG INDUCED ANTIGENIC REMODELING: PHARMACOLOGICAL BASIS TO DEVELOP A HIGHLY ACTIVE ANTI-TUMOR IMMUNOCHEMOTHERAPY

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Previous studies, performed in our laboratory, showed that chemotherapy can upregulate the expression of carcinoembryonic antigen (CEA) and thymidilate synthase (TS) in human cancer cells, thus increasing their immunogenicity. New drugs have been recently introduced in the treatment of colorectal carcinoma, therefore we decided to investigate the influence of gemcitabine (GEM, 500 μg/ml), leucovorin (L, 10^{-4} M), and 5-FU (10^{-5} M) on day 1 and oxaliplatin, (OXA, 10^{-4} M) on day 2, alone or in combination (GOLF) on CEA and TS expression in HT-29 colon cancer cells. CEA and TS protein expression was evaluated by cytofluorimetric and western blot analysis. Densitometric analysis of the immunoblot showed that CEA levels were 3.2, 2.4, and 3,8 fold higher in HT-29 cells treated with 5-FU, GEM and GOLF respectively compared to those of untreated cells. On the contrary, OXA alone did not influence CEA expression. However, when the cells were exposed to OXA before, instead of after 5-FU treatment, the up-regulation of CEA was partially inhibited. Furthermore, protein extracts from 5-FU treated cells showed the presence of two bands of 30 and 35 Kda, corresponding to TS free and ternary complex respectively. The amount of total TS expression (TS free + ternary complex) in cell extracts treated with 5-FU was two fold higher than that of untreated control. Moreover both cytofluorimetric and Western blot analysis showed that the enhanced expression of TS in the 5-FU treated colon carcinoma cells, was also detectable in GOLF-treated cells. Results of real time RT-PCR confirmed that CEA transcripts were 2.9, 2 and 1,6 times higher in HT-29 cells treated with 5-FU, GEM and GOLF respectively compared with those of untreated cells. These results suggest that OXA at the optimal treatment schedule (i.e. 24 h after exposure to 5-FU) does not reduce 5-FU-mediated CEA up-regulation. Our study provides the rational bases to design a protocol of chemo-immunotherapy, employing GOLF combined with host sensitization against CEA and TS-derived immunogenic peptides.

6 ANTI-PROLIFERATIVE EFFECT OF INTERLEUKIN-18 ON AN ORAL CARCINOMA CELL LINE

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Interleukin-18 (IL-18), a pro-inflammatory cytokine that is produced by both lymphoid and non-lymphoid cells, has a critical role in modulation of innate and adaptive immunity. Its primary function in stimulation of IFN-γ production, stimulation of NK cell cytotoxic activities and its adjuvant-like property make this cytokine a candidate for cancer immunotherapy. It has been shown that this cytokine is also produced by oral epithelia and carcinoma cells. A high serum level of IL-18 related to tumor regression in nude mice bearing salivary adenocarcinoma has been documented. However, direct effects of this cytokine on oral cancer cells have not been elucidated. In this project, we investigated IL-18 effect on an oral carcinoma (KB) cell line. With RT-PCR technique, KB cell line was found to express IL-18 receptors (IL-18Rα and IL-18Rβ) and their expression was influenced by IL-1β and TNF-α. This finding indicated that this oral carcinoma line is a target of IL-18. Recombinant human IL-18 inhibited KB cell proliferation by 17 percents at concentration of 100 ng/mL ($p<0.05$). Relative LDH release by these cells in treatment group and control groups was comparable, indicating that IL-18 suppression of cell proliferation was not mediated by the induction of cell death. To further address this hypothesis, we found that IL-18 treatment did not induce apoptotic cell death as studied by DNA laddering and TUNEL assays. In addition, expression pattern of cell death controlling genes (bcl-2 and bax) was not altered by this cytokine. Findings in these studies indicated that suppression of KB cell proliferation may be attributed to control of cell cycle, growth arrest or induction of cell differentiation. The data presented in this project could provide an insight of how cancer cell directly responses to IL-18 as this cytokine is an important regulator of anti-cancer mechanisms.

7 IMMUNIZATION OF C57/BL/6 MICE TRANSGENIC FOR HUMAN CEA WITH RECOMBINANT ADENOVIRAL VECTORS IN PRESENCE OF ADJUVANTS

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The carcinoembryonic antigen (CEA) is an attractive target for immunotherapy since it is expressed in many epithelial carcinomas and is involved in tumor progression. Immunization of C57/BL/6 mice (non-tolerant model) with recombinant adenoviral vectors carrying the human CEA c-DNA induced both anti-CEA T cell mediated immune and humoral immune responses. The same immunization schedule in C57/BL/6 mice transgenic for human CEA (tolerant model) induced anti-CEA T cell mediated immune response but very poor anti-CEA immunoglobulin production. To overcome tolerance and induce anti-CEA antibodies, the effect of adjuvants inducing dendritic cell (DC) maturation was explored. We have used two different adjuvants: Monophosphoryl Lipid A (MPL) acting on Toll-like receptor-4, and CpG motif-containing oligodeoxynucleotides (CpG-ODN) active on Toll-like receptor-9.

Firstly, we evaluated *in vivo* the trans-gene expression when the adenoviral vector was co-administered with the adjuvant and we did not observe reduction of the expression. Afterwards, the immune response was monitored and compared to that obtained in absence of adjuvant. The cell mediated immune response was measured by Cytokine Flow Cytometry (CFC) for IFN-gamma production upon stimulation with CEA pool of peptides containing sequences previously identified as immunogenic for the mice strain C57/BL/6. CD8 mediated IFN-gamma production was detected in all immunized groups independently from the use of the adjuvant. Anti-CEA antibodies (IgG) were measured by ELISA and significantly higher antibody titers were obtained in presence of either MPL or CpG ODN adjuvant as compared to the vaccination with the recombinant adenoviral vector alone. In summary, we have observed that antibody production against a self antigen can be restored in a tolerant model when the genetic vector used for vaccination is co-administered with the adjuvant acting on DC maturation.

8 EXPRESSION OF CD40 LIGAND (CD154) IN RECOMBINANT VACCINIA VIRUS: EFFECTS ON APC AND CTL PRIMING

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Recombinant poxviruses expressing immuno-modulatory molecules together with specific antigens might represent powerful vaccines for cancer immunotherapy. Recently, we and others have demonstrated, *in vitro* and in clinical trials, that co-expression of costimulatory molecules (CD80 and CD86) could increase the immunogenic capacity of a recombinant vaccinia virus (rVV) also encoding different tumor associated antigens. In order to further investigate the capacity of these vectors to provide ligands for different co-stimulatory pathways relevant in the generation of $CD8^+$ T-cell responses, we designed a recombinant virus (rVV) expressing CD40 ligand (CD154). This co-receptor, expressed on activated $CD4^+$ T cells, upon binding CD40 expressed on antigen presenting cells (APC) has been reported to increase their antigen presentation and immunomodulatory capacities.

To investigate the potency of CD154rVV in CTL generation, different types of infection were performed in cultures containing APC and $CD8^+$ cells. Phenotypic characterization of infected iDC showed that CD154rVV enhances their activation and maturation, measured by increased expression of CD83 and CD86, as compared to wild type vaccinia virus (WTvv). Cytokine gene expression was evaluated by quantitative real time PCR. As expected, WTvv infection triggered cytokine gene expression in APC and T-cells. However, typical APC cytokines such as TNFalpha and IL15 and, on the other hand, typical T-cells cytokines such as IL-2 and IFN-gamma seemed to be expressed to a higher extent in CD154rVV infected cultures. Furthermore, as a landmark of the CD40-CD154 pathway, IL12p40 gene transcription in iDC was exclusively induced by CD154rVV infection. The latter factor is also known to play a major role in CTL priming.

Activation of specific $CD8^+$ T-cells, was investigated by using the Mart-1(27-35) epitope as model antigen and monitored by tetramer staining and cytotoxic assays. We found that increased numbers of specific cytotoxic CD8 T-cells were induced by the specific peptide in the presence of the CD154rVV activated APCs, as compared to WTvv.

Taken together, these data indicate that functional CD154 expression from rVV infected cells induces APC activation and maturation thereby enhancing antigen specific $CD8^+$ T-cell generation. Such recombinant vector might help bypassing the requirement for activated helper cells thus qualifying as a potentially relevant reagent in the generation of $CD8^+$ T-cell responses in cancer immunotherapy.

9 INFLUENZA VIROSOMES ENHANCE CLASS I RESTRICTED CTL INDUCTION THROUGH CD4+ T CELL ACTIVATION

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The use of synthetic HLA class I restricted peptides in vaccination provides high specificity in targeting cytotoxic T cell responses. A major drawback, however is represented by their poor immunogenicity. Administration of peptides together with appropriate adjuvants might result in more effective immune responses.The search for adjuvants capable to enhance cellular immune responses is one major goal in vaccination against tumours and defined chronic microbial infections.

Immunopotentiating reconstituted influenza virosomes (IRIV) are one of the few adjuvants currently licensed for human use. While their adjuvant capacity in the induction of humoral responses is clearly documented, few data exist on their effects on T cell immune response. Here we addressed IRIV adjuvance in the induction of HLA class I restricted cytotoxic T lymphocytes (CTL) *in vitro*.

Lymphocyte stimulation with HLA-A0201 restricted influenza matrix peptide 58-66 (IM58–66) and IRIV resulted in marked expansion of specific CTL as compared to cultures performed in the presence of either peptide alone or peptide and control liposomes. Studies addressing underlying adjuvant mechanisms demonstrate that IRIV activate CD4/CD45RO$^+$ T cells, induce a cytokine profile consistent with T helper 1 (Th1) stimulation and increase the percentages of CD4$^+$ T cells expressing CXCR3. Furthermore, supernatants from IRIV stimulated PBMC cultures promote dendritic cell (DC) maturation. Importantly, IRIV mediated CTL adjuvance requires the presence of live CD4$^+$ T cells and appears to act mainly through secretion of soluble factors without an obligatory role for cell-cell contact. In relation to cancer powerful adjuvant effects of IRIV were also observed in the induction of CTL specific for the melanoma associated Melan-A/MART-127–35, HLA-A0201 restricted epitope.

Taken together these data indicate that IRIV are endowed with a high adjuvant capacity for HLA class I restricted CTL induction, largely attributable to their ability to antigenically stimulate CD4$^+$ T cells.

In vitro enhancement of tumour specific CTL induction by IRIV encourages further evaluation of this adjuvant for its possible use in cancer immunotherapy.

10 IMMUNIZATION OF STAGE IV MELANOMA PATIENTS WITH MART-1 AND GP100 PEPTIDES TOGETHER WITH IFN-ALPHA AS ADJUVANT RESULTS IN AN INCREASED NUMBER OF MONOCYTES/DENDRITIC CELL PRECURSORS

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Recent studies have shown that IFN-α induces the differentiation/activation of dendritic cells (DC) and acts as an adjuvant for the promotion of an antitumor immune response in mice. However, the use of IFN-ϙ as vaccine adjuvant has not been tested yet in clinical trials. We have carried out a pilot phase I-II trial to determine the adjuvant activity of IFN-α administered in combination with MART-126-35(27L) and gp100209-217(210M) peptides in HLA-A2 stage IV melanoma patients. The regimen consisted of two rounds of four vaccinations: 1) peptides given i.d. every two weeks and IFN-α (3 MU) s.c. on days –1, 0 and +1 with respect to peptides (day 0); 2) peptides given monthly with a single IFN dose (3 MU). Primary endpoints were evaluation of peptide specific $CD8^+$ T cells, phenotype and function of DC and treatment tolerability. Six out of the 9 patients enrolled completed at least the first vaccination round. IFNγ-ELISPOT on PBMC or purified $CD8^+$ T cells detected an enhancement of class I-HLA-restricted recognition of modified and native MART-1 and gp100 peptides in 2 patients, and an increased recognition of MART-1-derived peptides in one additional case. A simultaneous raise in $CD8^+$ HLA/MART-1 tetramer+ T cells was also observed in 2 of these 3 patients. Clinically, two patients showed stable disease and four underwent progression. One day after IFN/peptide vaccine administration, we evaluated the phenotype and functions of monocytes/DC in PBMC samples. In all patients, the percentage of $CD14^+$ monocytes augmented after treatment, with the fraction of $CD14^+/CD16^+$ cells being especially increased. In 5 patients, there was an evident augment in the percentage of $CD14^+/CD2^+$ and $CD14^+/CD83^+$ subsets. The percentage of $CD14^+$ monocytes expressing the CD40 and CD86 costimulatory molecules was also enhanced. In 2 patients showing stable disease, we evaluated the allostimulatory activity in MLR assays of post-vaccination monocyte derived-DCs, generated after a 24-h culture, and found that these cells exhibited a higher APC activity with respect to cells from pre-vaccination samples. These results are consitent with the increases in $CD14^+/CD2^+$ and $CD14^+/CD16^+$ monocyte subsets, considered as circulating DC or DC precursors, and suggest that the administration of the IFN-α/peptide vaccine can increase *in vivo* the frequency of active APCs. Studies with larger number of patients and based on different vaccine formulations would be needed to evaluate the possible adjuvant effect of IFN-α on tumor-specific $CD8^+$ cells and the correlation between changes in the phenotype/function of circulating DC/DC precursors and the possible clinical response.

11 MONOCLONAL ANTI-MAGE-3 CTL RESPONSES IN MELANOMA PATIENTS DISPLAYING TUMOR REGRESSION AFTER VACCINATION WITH A RECOMBINANT CANARYPOX VIRUS

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We have analyzed the T cell responses of HLA-A1 metastatic melanoma patients with detectable disease, following vaccination with a recombinant ALVAC virus, which bears short MAGE-1 and MAGE-3 sequences coding for antigenic peptides presented by HLA-A1. To evaluate the anti-MAGE CTL responses, we resorted to antigenic stimulation of blood lymphocytes under limiting dilution conditions, followed by tetramer analysis and cloning of the tetramer-positive cells. The clones were tested for their specific lytic ability and their TCR sequences were obtained. Four patients who showed tumor regression were analyzed, and an anti-MAGE-3.A1 CTL response was observed in three of these patients. Post-vaccination frequencies of anti-MAGE-3.A1 CTL were $3x10^{-6}$, $3x10^{-3}$, and $3x10^{-7}$ of the blood CD8 T cells, respectively. These three responses were monoclonal. No anti-MAGE-1.A1 CTL response was observed. These results indicate that, like peptide immunization, ALVAC immunization produces monoclonal responses. They also suggest that low-level anti vaccine CTL responses can initiate a tumor regression process. Taken together, our analysis of anti-MAGE-3.A1 T cell responses following peptide or ALVAC vaccination shows a degree of correlation between CTL response and tumor regression, but firm conclusions will require larger numbers.

12 IMMUNIZATION OF MICE TRANSGENIC FOR HUMAN HLA-DR4 WITH HUMAN CEA REVEALS NOVEL HUMAN CEA EPITOPES FOR T HELPER LYMPHOCYTES

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The human carcinoembryonic antigen (CEA) provides a relevant tumor self-antigen target for a vaccine. Although many epitopes capable of inducing CTLs have been characterized, only few class II restricted T helper epitopes have been so far identified.

Genetic immunization of C57BL/6 mice transgenic for human HLA-DR4 (C57/DR4) has been performed with DNA coding for human CEA tumor antigen to experimentally find CEA epitopes binding to human MHC class II. Spleen cells from immunized animals have been analyzed by ELISPOT for IFN-gamma production upon stimulation with pools of overlapping peptides (15 mer overlapping by 11) covering the entire CEA sequence. Individual reactive peptides have been mapped and the subset of reactive lymphocytes identified by Cytokine Flow Cytometry (CFC). Four different epitopes for CD4 lymphocytes were found. Two of the four identified regions correspond to CEA CD4 immunogenic sequences previously predicted by algorithms and found to be immunogenic in humans. Two additional sequences were identified using our experimental approach. *In vitro* priming of CD4 T cells from human healthy donors using synthetic peptides corresponding to mapped regions is now in progress. Donors have been characterized for their DR type to experimentally characterize for promiscuity the identified CD4 epitopes. Moreover, individual peptides corresponding to the mapped regions have been used for vaccination of C57/DR4 mice to explore their immunogenic potential *in vivo*. The knowledge of both CD8 and CD4 specificities will strongly facilitate the development of a therapeutic vaccine based on the use of synthetic peptides capable of inducing both CTL and T-helper immune responses.

13 IMMUNIZATION WITH GENETIC VECTORS EXPRESSING RHESUS CEA EFFICIENTLY BREAKS IMMUNE TOLERANCE IN MICE AND RHESUS MONKEYS

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CEA is a 180KDa glycoprotein over-expressed in a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast and lung. For this reason, it is currently under evaluation in clinical trials as target for immunotherapy in the treatment of colorectal cancer.

To demonstrate that genetic vaccination with vectors expressing this tumour antigen is capable of specifically breaking immunotolerance in non-human primates, it is necessary to use the equivalent of human CEA. Since the rhesus monkey (macaca mulatta) homologue of this human tumour associated antigen was not available, we have identified and cloned rhesus CEA (rhCEA) from colon tissue samples. rhCEA is an open reading frame of 2118 nucleotides encoding a 705 aa polypeptide with 78.9% homology to human CEACAM-5 protein.

Vaccination protocols using rhCEA expressing vectors were designed both for mice and rhesus monkeys. CEA.Tg mice are transgenic mice that express human CEA with a tissue distribution similar to that of humans. To demonstrate the capability of xenogeneic vaccination to elicit an immune response against CEA as self-antigen in this model, we immunized CEA.Tg mice with vectors encoding either human (homogeneic) or rhesus CEA (xenogeneic).

After treatment of mice with DNA followed by EGT (Electro Gene Transfer) and adenovirus boosting, cross-reactive antibodies against human CEA protein were measured only in rhesus CEA immunized groups. Importantly, cellular immune-response against human CEA was observed upon immunization with rhesus CEA both in wild type and transgenic mice.

To assess the efficiency of immunization of rhesus macaques with rhesus CEA, four monkeys were immunized with DNA+EGT followed by adenovirus injection. The cell mediated response was measured by IFNγ Elispot assay and the humoral response was measured by ELISA assay. We measured cell mediated immune responses in two treated monkeys and three of them showed a good anti-CEA antibody titer, ranging from 1:143 to 1:2099.

These data show that genetic vectors encoding for rhCEA are able to break the immune tolerance to this tumour antigen in mice as xenogene and in primates as self-gene involving both cell mediated and humoral arm of the immune response.

14 GENE OPTIMIZATION OF RHESUS CEA FOR MONKEY IMMUNIZATION

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Codon usage optimization is a tool to develop expression cassettes for genetic vaccination in which antigens are more efficiently expressed and ultimately lead to a greater immune response. This requirement is of particular relevance when dealing with self tumour antigens such as CEA against which the immune response is difficult to elicit since tolerance must be broken to activate the immune system.

We have constructed a synthetic codon usage optimized cDNA of rhesus monkey (macaca mulatta) Carcinoembryonic antigen (rhCEA). The coding region of the cDNAopt is 2118 nucleotides long and it encodes a protein of 705 amino acids. Transfection with an expression plasmid (pVIJ-rhCEAopt) or infection of HeLa cells with a first generation ΔE1-ΔE3 adenovirus vector (Ad5-rhCEAopt) carrying the cDNAopt of rhesus CEA (rhCEAopt) showed 10-50 fold greater protein levels than a similar vector carrying the native cDNA. Similarly, intramuscular injection of pVIJ-rhCEAopt followed by EGT (Electro Gene Transfer) or Ad5-rhCEAopt vector in CEA transgenic mice resulted in greater protein levels than those detected upon injection of vectors encoding for rhCEA. Mice immunized with plasmid/ adenovirus vector mixed modality, both containing the cDNAopt showed strong cross reactive human CEA-specific antibody response, 300-fold higher than hCEA containing vectors. Cell mediated responses were two- and three-fold higher against rhesus or human protein, respectively, than using the vectors containing the native rhCEA. These data show that rhCEAopt is efficient to induce a human CEA cross-reactive response and to break immune tolerance, in CEA transgenic mouse model.

To verify the efficacy of gene optimization in rhesus monkeys, we injected vectors encoding for rhCEAopt in eight monkeys. Both Ad vectors alone or in combination with DNA were efficient in breaking immune tolerance to CEA in immunized rhesus monkeys and maintain over time elicited immune response.

Our data show that use of rhesus CEA and development of modified expression cassettes that result in increased potency of Adenovirus, plasmid DNA and other gene delivery vaccine approaches may have significant impact on vaccine development of neoplastic malignancies expressing CEA.

15 ENHANCED IMMUNOGENICITY OF CODON OPTIMIZED CDNA OF HUMAN CARCINOEMBROYNIC ANTIGEN

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Carcinoembryonic antigen (CEA) is a 180KDa glycoprotein over-expressed in a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast and lung and thus constitutes an attractive target for cancer immunotherapy. To enhance the immunogenic properties of genetic vaccines expressing CEA, a synthetic cDNA of human CEAn optimized for codon usage has been constructed and characterized. Infection of HeLa cells with a ΔE1-ΔE3 adenovirus vector (Ad5-hCEAopt) carrying the cDNAopt of CEA showed 10 fold higher protein expression than vector Ad5-hCEA that carries the native cDNA of this target antigen. A similar increase in protein expression was detected in serum of C57Bl/6 mice injected i.m. with Ad5-hCEAopt.

Mice immunized with Ad5-hCEAopt elicited CEA-specific antibody and cell mediated responses at a dosage 10 fold lower than that required for Ad-hCEA. Similarly, mice electro-injected with 5 μg of plasmid pVIJ/hCEAopt showed a cell mediated response that was 3 fold higher than that obtained with plasmid pVVIJ/hCEA. Finally, immunization of CEA transgenic mice based on the combined use of plasmid and Ad vectors indicates that vaccination protocols based on the use of codon optimized cDNA are more efficient in breaking tolerance to the target antigen. Thus, these results demonstrate that codon optimized cDNA of CEA is an improved immunogen for genetic vaccination protocols.

16 EXPRESSION AND IMMUNOGENICITY OF CANCER/TESTIS TUMOR ASSOCIATED ANTIGENS IN NSCLC

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Cancer/testis tumor associated antigens (C/T TAA) might represent relevant targets for specific immunotherapy in non small cell lung carcinomas (NSCLC). Here we comparatively investigated their expression levels and specific immune responsiveness in freshly excised NSCLC specimens, by quantitative real time PCR and by peptide stimulated CTL induction *in vitro* from the corresponding tumor infiltrating lymphocytes (TIL), respectively.

Surgically removed NSCLC were mechanically disrupted to single cell suspensions that were either cultured in presence of IL-2 and solid phase bound anti CD3 to expand TIL or used to isolate total cellular RNA. MAGE 1, 2, 3, 4, 10, 12 and NY-ESO-1 gene transcripts were amplified by real time PCR following reverse transcription. The induction of specific CTL for MAGE 1, 3 (HLA-A1 restricted epitopes) and MAGE 4, 10, NY-ESO-1 and an epitope in common between MAGE-2-4-6-10 and 12 - multi-MAGE - (HLA-A2 restricted) was assessed upon stimulation of TIL with autologous peptide pulsed mature dendritic cells (mDC) as APC. Specific responsiveness was evaluated in standard 51Cr release assays.

Twelve specimens (7 adenocarcinoma and 5 squamous cell carcinomas) were tested and C/T TAA expression was detected in 2 adenocarcinomas and in 1 squamous cell carcinoma samples. Importantly, in these cases the expression of different C/T TAA was simultaneously detectable in clusters. Seven TIL populations could be expanded in culture. After four rounds of restimulation, weak (<20% specific killing) CTL responses specific for C/T MAGE 3 or 4 TAA (HLA-A1 and -A2 restricted, respectively) were only observed in two cases without obvious correlations with TAA expression.

Altogether, these preliminary data underline the difficulty to detect pre-existing C/T TAA specific immunity in NSCLC patients. Possibly, synthetic peptides represent ineffective immunogenic materials for CTL expansion *in vitro*. Ongoing generation of a recombinant vaccinia virus encoding MAGE 2, 4, 10, Multi-MAGE, NY-ESO-1 epitopes together with CD80 and CD86 costimulatory molecules could result in enhanced C/T TAA specific CTL generation.

17 GENE EXPRESSION IN CLASSICAL MIDGUT CARCINOID TUMORS: VMAT1 AND GAGE ANTIGENS ARE POTENTIAL TARGETS FOR CARCINOID IMMUNOTHERAPY

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This study aimed to identify genes preferentially expressed in classical midgut carcinoid tumors in an effort to identify potential target antigens for carcinoid immunotherapy. Classical midgut carcinoids are rare tumors originating from the enterochromaffin cells in the jejunum, ileum, cecum and ascending colon. The gene expression profile of midgut carcinoid tumors is unknown. This is partly due to the fact that midgut carcinoids are extremely rare and that it is difficult to obtain sufficient amounts of normal neuroendocrine cells from the gastrointestinal tract as controls for microarray analysis. We analyzed the expression of 34 genes by reverse transcription-PCR in a panel of 15 midgut carcinoid specimens and in the human endocrine pancreatic tumor cell line BON. Genes expressed in midgut carcinoids were also investigated for expression in up to 26 normal tissues. Our results show that the vesicular monoamine transporter 1 (VMAT1) is highly expressed by midgut carcinoids, both at the mRNA and protein level. VMAT1 expression in normal tissues is limited to enterochromaffin cells in the hollow gastrointestinal tract, to chromaffin cells in the adrenal gland and to cells in the substantia nigra of the brain. GAGE genes (GAGEs), which are known to be expressed by normal testis and various tumor tissues, are also expressed in midgut carcinoid tumors. In addition, we detected minor GAGEs expression in normal pancreas and stomach, which has not previously been described. Due to their limited expression in normal tissues, VMAT1 and GAGEs may be appropriate targets for T cell-based immunotherapy. Our study is the first to analyze the expression of potential midgut carcinoid target genes in a broad set of tumor specimens and normal tissues, and it represents the first step to antigen-specific immunotherapy of midgut carcinoid tumors.

18 TUMOR PROTECTION AND IMMUNORESPONSE IN MICE VACCINATED WITH HUMAN EP-CAM BUT NOT WITH THE MOUSE-EP-CAM HOMOLOGUE

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The human colorectal carcinoma (CRC)- associated antigen, named Ep-CAM, CO17-A, KSA and GA733 has been the target of passive immunotherapy using low affinity monoclonal antibody. This approach, which is based on the induction of an ADCC response, showed prolong survival of stage III CRC patients. Recently a paper reported the potential use of Ep-CAM as target for cell mediated immunoresponse induced by ALVAC vaccination in CRC patients. To shed light on the potential use of this antigen in experimental animal models we explored the potential use of adenovirus vector as delivery vehicles for Ep-CAM vaccination. A prime boost protocol was used to induce a $CD3^+/CD8^+$ IFNγ response (measured by ICS) against the non self human antigen, which could protect mice from a lethal injection of cancer cells. Unfortunately, in the same experimental condition, mouse Ep-CAM vaccination did not induce either an immunoresponse or a protection against a tumor challenge. A sporadic $CD3^+/CD8^+$ IFNγ response (20% of responders) was observed only in out-bread CD1 mice. These experiments indicate that the use of a potent vaccine vector is not sufficient to break immunological tolerance against the self Ep-CAM antigen.

We are currently evaluating the use of IL-2 as adjuvant in adenovirus vaccination.

19 PRIMING WITH FILAMENTOUS BACTERIOPHAGE fd VIRIONS EXPRESSING MAGE-A10 OR MAGE-A3 PEPTIDES INDUCES THE GENERATION OF AN EFFECTIVE ANTI-TUMOR CYTOTOXIC T-LYMPHOCYTE ACTIVITY

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Delivery of tumor-associated antigenic (TAA) peptide in a high immunogenic form represents one of the key issues for effective peptide-based cancer vaccine developement. MAGE TAA peptides elicit considerable interest because they are tumor-specific and expressed with high frequency in a variety of tumors. Here, we report the ability of non-pathogenic filamentous bacteriophage fd virions to deliver HLA-A2-restricted MAGE-A10- or MAGE-A3-derived peptides and elicit potent specific cytotoxic T-lymphocytes (CTL) responses *in vitro* and *in vivo*. Based on a modification of the phage display technology, we constructed double-display fd23/Mg10 and fd23/Mg3 virions, coexpressing the promiscuous HLA-DR-restricted Th peptide (pep23) from HIV-1-RT, and the HLA-A2-restricted CTL nonapeptide-encompassing residues 254-262 (Mg10) or 271-279 (Mg3) from MAGE-A10 and MAGE-A3 TAA, respectively. We showed that a single *in vitro* stimulation of human peripheral blood mononuclear cells with monocytes pulsed with fd23/Mg10 or fd23/Mg3 virions and a single immunization of HHD (HLA-A2 transgenic) mice with fd23/Mg10 or fd23/Mg3 particles elicited potent helper T-cell-dependent specific CTL responses. Notably, Mg3 peptide-specific CTL were able to kill HLA-A2 positive/MAGE-A3 positive tumor cells, known to be not recognized by peptide-specific CTL generated by conventional stimulation procedures. Overall our results show the efficacy of this TAA peptide delivery system to elicit specific CTL responses which appear to be remarkably potent. These data indicated that engineered filamentous bacteriophage virions increased substantially the immunogenicity of delivered TAA peptides, thus representing a novel powerful system for the developement of effective peptide-based cancer vaccines.

20 CHARACTERIZATION OF CELLULAR LOCALIZATION AND TRANSPORTATION OF A NOVEL COLORECTAL TUMOR ASSOCIATED ANTIGEN (COA-1) THAT CAN ELICIT TUMOR SPECIFIC IMMUNE RESPONSE

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COA-1 is a novel colorectal tumor associated antigen recognized by $CD4^+$ T lymphocytes in association with HLA class II molecules (DRβ1*0402 or DRβ1*1301) (Maccalli et al., Cancer Res 63:6735, 2003). The nucleotide sequence of COA-1 transcript was nearly identical to multiple expressed sequence tag sequences encoding variants of Socius, a protein that was found recently to bind to members of the Rnd family GTPases. The COA-1 gene was expressed, as shown by RT-PCR analysis, at relatively comparable levels in colorectal and melanoma tumor cells, EBV-infected B cells, normal B cells and fibroblast cell lines. However, the gene isolated from normal cells contained a single nucleotide substitution, leading to an amino acid change in the C terminal protein region. Although the minimal epitope recognized by $CD4^+$ cells was encoded by sequences located upstream from the base substitution, the expression of sequence variants correlated with the selective recognition of tumor by cells T lymphocytes. This evidence suggested that differential cellular localization and/or processing of the antigen can occur in malignant and normal cells. With the aim of investigating this issue, laser scanning confocal microscopy analysis has been performed on a panel of normal and tumor cell lines using a specific polyclonal antibody directed to COA-1. The intra-cellular localization and the translocation pathway to the cellular membrane of COA-1 has been studied, with a particular attention to the protein association with cytoplasmic organelles and cytoskeleton structures. A co-localization of the protein with Golgi apparatus and with the vesicles mediating cellular transportation to the plasma membrane has been detected; in addition, intracellular association of COA-1 with one of the microtubule components, tubulin, or with HLA class II molecules occurred. Interestingly, nuclear localization of COA-1 was detected selectively in tumor cells, indicating that, indeed, differential localization of this protein occurred in normal and malignant cells. Further studies are currently ongoing to determine COA-1 intra-cellular localization and the pathway of transportation. We can conclude that the differential localization of the protein could affect the presentation on cellular surface, in association with HLA molecules, of COA-1-derived immunogenic epitopes, thus resulting in the antigen capability of raising a tumor specific immune response. These results represent a first step in understanding the biological function of this novel tumor associated antigen. We suggest that this protein could represent a marker for transformation and/or progression of colorectal cancer as well as a potential candidate for the development of cancer vaccines.

21 LIMITED CROSS-RECOGNITION OF NATIVE PEPTIDE AND HLA-A*0201^{+}CEA^{+} COLON CARCINOMA CELLS BY CD8^{+} T CELLS RAISED IN THE PRESENCE OF THE SUPERAGONIST CEA-ANALOGUE CAP1-6D

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One strategy for boosting anti-tumor T cell-mediated immune responses is represented by the use of "altered peptide ligands" (APL), i.e. peptide analogues characterized by improved immunogenicity as compared to native epitopes expressed by cancer cells. In the present study we characterized the immunogenicity of CAP1-6D, a superagonist analogue of the carcinoembrionic antigen (CEA)-derived HLA-A*0201-restricted epitope CAP1. This epitope was obtained by introducing an Asp in position 6 (YLSGADLNL) and described to potentiate CEA-specific T cell responses through increased binding to T cells. To evaluate the potential usage of CAP1-6D in the ex-vivo generation of CEA-specific T cells for adoptive transfer, PBMCs obtained either from healthy donors (n=6) or colon carcinoma patients (n=3) were stimulated *in vitro* with CAP1-6D-pulsed autologous DCs, and then tested for the ability to cross-react with the native peptide CAP1 and to recognize CEA^{+} HLA-A*0201^{+} colon carcinoma cells, by assessing specific cytokine release, cytolytic activity and HLA tetramer binding. In all cases analyzed, anti-CAP1-6D T cells were found to recognize the native peptide CAP1 with reduced and variable efficiency. Cold-target inhibition assays and TCR down-modulation experiments showed that only a limited subset of T cells raised in the presence of the superagonist peptide were indeed able to interact with the natural CAP1 epitope, and only under saturating experimental conditions. Moreover, anti-CAP1-6D T cells secreted IFN-γ, but not IL-2 and TNF-α in response to CAP1, suggesting a potential role of the native peptide as partial agonist or antagonist of these T cells. As for cross-recognition of tumor cells, anti-CAP1-6D T cells were unable to effectively recognize HLA-A*0201^{+}/CEA^{+} colon carcinoma cells which, on the contrary, were able to trigger IFN-γ release by T cells generated with the natural epitope CAP1. Altogether, our data show that the superagonist peptide CAP1-6D induces the *in vitro* expansion of specific CD8^{+} T cell with limited ability to cross-react with native peptide, especially when endogenously presented by CEA^{+} colon carcinoma cells, and underline the need for extensive immunological analysis of T cell reactivities raised by APL of tumor antigens in terms of ability to recognize natural epitopes expressed by cancer cells. (supported by Italian Association for Cancer Research).

22 INCREASED NK ACTIVITY IN COLON CARCINOMA PATIENTS VACCINATED WITH AUTOLOGOUS TUMOR-DERIVED HSP96

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We recently showed that vaccination with autologous tumor-derived Heat Shock Protein (HSP) gp96 (HSP96) increased $CD8^+$ T cell-mediated anti-tumor responses in patients radically resected for liver metastases from colo-rectal cancer. Here we report that treatment with HSP96 additionally causes a significant boost of NK activity in peripheral blood lymphocytes, as detected by cytokine secretion and cytotoxicity in the presence of NK-sensitive targets. Increased NK activity was associated to a raise in $CD3^-CD56^+$ and/or $CD3^+CD56^+$ cells, displaying enhanced expression of the stimulatory receptors NKG2D (in both $CD3^+CD56^+$ and $CD3^-CD56^+$cells) and NKp46 (in $CD3^-CD56^+$). $CD3^+CD56^+$ T cells expanded upon vaccination expressed a $CD3^+CD8^+TCR\alpha\beta^+$ phenotype and stained negative for TCRγδ and NKT (such as TCR Vα24Vβ11) markers. $CD14^+$ monocytes from PBMCs of vaccinated patients displayed up-regulated expression of CD83 and CD40, and enhanced release of IL-12 upon stimulation, suggesting that NK activation could occur through a monocyte-mediated indirect mechanism. Moreover, monocyte-depleted $CD56^+$ PBMCs from colon carcinoma patients exerted increased recognition of NK-sensitive targets and displayed up-regulated NKG2D and NKp46 expression when incubated with tumor-derived HSP96. These HSP96-activated cells mediated also significant lytic activity on colon carcinoma cells. Additionally, a limited but reproducible proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells from colon carcinoma patients (4%-9%, 2-12%, respectively) specifically bound FITC-conjugated HSP96, with a pattern highly resembling CD91 expression in these cell subsets. Altogether, these results suggest that HSP96 can enhance NK activity in NK and T cells through both indirect and direct mechanisms. Thus, HSP96 appears to mediate pleiotropic anti-tumor immune responses involving, in addition to antigen specific T lymphocytes, cellular component of the innate immunity.

23 TUMOR-SPECIFIC IMMUNITY BY A DNA VACCINE ENCODING A MODIFIED HPV16 E7 TUMOR ANTIGEN LINKED TO THE COAT PROTEIN OF A PLANT VIRUS

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The HPV16 E7 oncoprotein is expressed consistently in genital tumours and is responsible for the onset and maintenance of the transformed state. The E7 protein represents a valid target for the development of therapeutic vaccines against cervical malignancies.

DNA vaccination is an attractive approach for HPV immunotherapy and various strategies to enhance long-term humoral and cellular immunity are currently under active investigation. Clinical trials are also in progress based on the E7 oncogene fused to several activating sequences.

The goal of our research is to evaluate the efficacy, as therapeutic vaccines, of plasmids carrying the E7 gene fused to genes from plant viruses or other plant-derived sequences. The basic idea is that, besides their exploitation as biofactories, plants and plant viruses can offer new opportunities to increase the efficacy of DNA vaccines by providing intrinsically safer sequences that are primary antigens in humans.

In this perspective we firstly focused on the Potato Virus X coat protein (PVX-CP). This protein, with its intrinsic self-assembling ability (leading to highly aggregated structures in cell cultures) has already shown to be able to increase the $CD4^+$ T- cell mediated immune response against B-cell malignancies in a mouse model.

Because of safety concerns, the direct use of an oncogene is impossible in human vaccination. Therefore, we firstly introduced three point mutations into the pRb-binding site of HPV16 E7 gene to abolish its transformation potential ("E7GGG" gene). The modified E7GGG sequence was fused in frame to the fourth codon of the PVX-CP, either directly or via the flexible amino acid linker "Gly-Pro-Gly-Pro". The synthetic genes were cloned into a mammalian expression vector.

Both vaccine preparations, when inoculated in C57/BL6 mice bearing E7 protein-expressing tumours, elicited an immune response and induced regression of the neoplasia, showing their efficacy as therapeutic vaccines.

24 A THERAPEUTIC VACCINE AGAINST HPV-ASSOCIATED NEOPLASTIC LESIONS BASED ON CRUDE PLANT EXTRACTS CONTAINING HPV16-E7

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Plants are increasingly being employed for the production of clinically important recombinant proteins. Before starting a plant-based production of a vaccine, prior studies of therapeutic effectiveness with conventional production procedures as well as established protocols for animal vaccination must be available if transition to human application has to be performed.

We focused our attention on the human papilloma virus (HPV), the primary causative agent in more than 90% of cervical cancer, with HPV16 being the type most frequently found in these tumors. The E7 viral protein is highly expressed in HPV-associated cervical cancers, representing the target of choice for therapeutic vaccination. Several clinical trials based on the HPV16 E7 (i.e. as DNA vaccines or as recombinant protein) are in progress.

We have reported the production of HPV16 protein in Nicotiana benthamiana tobacco plants using a plant expression vector derived from the Potato Virus X (PVX). Mice immunized with crude extracts containing E7 (Nb-PVXE7) showed stimulation of humoral and cell-mediated immune responses, suggesting an adjuvant-like activity of the foliar extracts. After challenge with E7-expressing C3 tumor cells, tumor growth was completely inhibited in 40% of the animals, while the remaining mice showed a drastic reduction of tumor size. The amount of the E7 protein in the extract was 20-fold lower than a preparation known to prevent tumor growth in all the animals, based on purified His-E7 expressed in E. coli (10 micrograms/booster) plus the adjuvant Quil-A.

In an attempt to improve the vaccination protocol, we tried to increase E7 protein expression levels. This also represents a necessary step before adopting the plant system as a cost-effective way for large-scale production of an E7-based anti-cancer vaccine. A secretory version of the E7 gene was achieved by using a plant-derived signal sequence and cloned in the PVX-derived vector. Targeting to the plant secretory pathway enhanced the amount of the E7 protein produced (about five-fold). Mice immunised with the new plant extract showed a fast and increased immune response and a better tumor protection.

25 VACCINATION STRATEGIES AGAINST LONG-TERM LATENT GAMMAHERPESVIRUS INFECTION IN THE MODEL OF MURINE HERPESVIRUS 68 (MHV-68)

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Human gammaherpesviruses such as Epstein-Barr virus cause lifelong infections and associated diseases, including malignancies, by virtue of their ability to establish latent infection.

The MHV-68 model provides a useful experimental setting to evaluate the efficacy of vaccination strategies against gammaherpesvirus infections. We reported that vaccination with heat-inactivated MHV-68 protected immunocompetent mice from the acute and latent MHV-68 infection, and saved highly susceptible mice from a lethal infection with the virus. However, heat-inactivated MHV-68 failed to prevent the establishment of a long-term latent MHV-68 infection in the spleen.

In order to investigate whether a live-attenuated MHV-68 vaccine could elicit a memory immune response protective against long-term latency, we isolated and characterized a clone of recombinant MHV-68 carrying the mouse IFN-α1 coding sequence (MHV-68mIFNα1). Although the *in vitro* replication of MHV-68mIFNα1 was not affected by the production of high amounts of the cytokine by the infected cells, MHV-68mIFNα1 was severely attenuated *in vivo*. In particular, two months after the infection with MHV-68mIFNα1 the number of latently infected splenocytes was virtually undetectable, whereas long-term latent infection was clearly present in mice infected with wt MHV-68. Notably, neither sign of virus acute replication, neither peak nor long-term latency was observed when mice were injected with MHV-68mIFNα1 partially inactivated upon treatment with psoralen and UV. These results prompted us to compare the efficacy of live-attenuated vs. partially inactivated MHV-68mIFNα1 in a prophylactic vaccination regimen aimed at inhibiting the establishment of MHV-68 long-term latency after the infection. The results of these ongoing experiments will be presented.

As a further approach to prevent long-term consequences of gammaherpesvirus infection, we tested the efficacy of a combination therapy based on the treatment of mice latently infected with MHV-68 with a chemotherapic agent followed by the adoptive transfer of cells immune to MHV-68. To this end, mice chronically infected with MHV-68 were given cyclophosphamide a few hours before the infusion of spleen lymphocytes isolated from mice vaccinated with inactivated virus. Interestingly, the majority of the treated mice showed the complete remission of latency, indicating the superior efficacy of the combined chemo-immunotherapy approach as compared to any therapeutic vaccination strategy attempted to date.

26 XENOGENIC TUMOR CELLS AS A LIVE ANTITUMOR VACCINE

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Up to date, live, viral and bacterial vaccines have proved to be efficient and reliable tools for immunoprophylaxis of infectious diseases. For example, smallpox was eradicated in the 20th century owing to immunization of human beings with a live vaccine - cowpox vaccinia virus, whereas morbidity from tuberculosis was significantly reduced through application of the live BCG vaccine prepared from the mycobacterium of bovine tuberculosis (M.bovis).

Until recently, the use of live cell vaccines in oncology was restricted due to several reasons, primarily because allogenic and autologous tumor cells are harmful for the recipient, especially with regard to virus-induced tumors, whereas xenogenic tumor cells are rapidly destroyed within human organism.

We made an attempt to implement vaccination with xenogenic tumor cells implanted into a connective tissue capsule formed by inert polyacrylamide gel preinjected under the skin of recipients. Such gel is widely used in plastic surgery for correction of small defects of muscle and connective tissue and has proved to be a safe biocompatible material. During 1–2 months, the gel was encapsulated and could serve as a medium for proliferating tumor cells. As a result, xenogenic tumor cells form solid tumors which are further rejected due to the formation of a sufficiently strong immune response. Animal studies have demonstrated a significant protective effect of human tumor cells (melanoma and adenocarcinoma) against syngenic transplantable tumors of C57Black/6 mice, melanoma B-16 and adenocarcinoma Ca-755, respectively.

Human tumor cells are innocuous for mice and most probably vice versa. Transplantable mouse tumors can easily be tested for the presence of pathogenic microflora. Therefore, transplantable mouse tumors are highly recommended as efficient vaccinating preparations against human melanomas and adenocarcinomas. Immunogenecity and safety of such vaccines was demonstrated in experiments with healthy volunteers. The usefulness of mouse melanoma B-16 for prevention of post-surgical metastatic growth and tumor recurrencies in melanoma patients is under II stage of clinical trials.

The antigenic similarity of many animal tumors to human tumors combined with pronounced interspecific histoincompatibility makes xenogenic tumors promising candidate materials for the design of novel anticancer vaccines.

27 PREVENTIVE VACCINATION AND INDUCTION OF MELANOMA-SPECIFIC CTL AFTER THE TRANSPLANTATION OF XENOGENIC TUMOR CELLS INTO THE CONNECTIVE TISSUE CAPSULE FORMED AROUND THE BIOCOMPATIBLE GEL

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Xenotransplantation of different cells into the soft biocompatible gel surrounded by connective tissue capsule provides their long survival rate inside the capsule. After introduction in such gel of the xenogenic tumor cells this phenomenon can be utilized for an induction of the specific immune response and antitumor vaccination. The opportunity of the specific antitumor response induction was shown in experiments on mice. A lot of mouse lymphocytes and alive tumor cells were found inside the gel in 1-4 weeks after the introduction of a human melanoma cells line SK-MEL-1 under a connective tissue capsule formed around of a gel. Very high cytotoxic activity (CTA) of lymphocytes extracted out of a capsule with a culture medium against syngenic cells of a mouse melanoma line B16 was found. The appearance of CTA of splenocytes against the same target cells B16 was simultaneously recorded. High antitumor effect was estimated when mouse melanoma line B16 was inoculated to syngenic mice in 4 weeks after the xenotransplantation of human melanoma cells. The inhibition of tumor growth and augmentation of lifespan of immunized animals was seen. At the same time after the intramuscular xenotransplantation the antitumor effect was absent.

Successful xenovaccination in gel is determined, apparently, by much longer physiological functioning of alive and release into the host bloodstream of the products of dead xenogenic tumor cells and due to this by longer secretion of species unspecific conservative tumor antigens, than it is after intramuscular xenovaccination. Such approach can be used in preventive vaccination against broad spectrum of human cancers.

28 NOVEL, CHIMPANZEE SEROTYPE-BASED ADENOVIRAL VECTOR AS VACCINE FOR CEA

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Adenovirus is an attractive vector for genetic vaccine development for its capability to infect several tissues and antigen presenting cells. This property is impaired by pre-existing immunity to the human adenoviruses in most of the population. For this reason, use of uncommon alternative Ad serotypes could overcome this problem.

Recently, chimpanzee Ad vectors have been shown to be efficient delivery tools for vaccination protocols.

To verify the capability of a chimp Ad vector to vaccinate hosts, an E1,E3 deleted recombinant adenovirus based on the chimpanzee serotype 3 (chAd3) was engineered to express human CEA protein.

CEA.Tg are transgenic mice that express human CEA with a tissue distribution similar to that of humans. CEA.Tg were intramuscularly immunized with this vector (chAd3hCEAopt) and expression of human CEA was monitored in the serum. Development of a CEA-specific CD8 immune response was observed after a single injection and it was comparable in terms of efficacy and kinetics to that of mice immunized with a similar vector based on human serotype 5 (Ad5hCEAopt) expressing the same transgene. Importantly, the capability of chAd3hCEAopt to elicit the immune response against CEA was not abrogated in animals pre-exposed to human wt Ad5 and works as booster of immunity elicited by other Ad vectors.

In conclusion, our data show that this novel chimp Ad based vaccine vector is able to induce an immune response comparable to Ad5 human serotype and, importantly to break tolerance to a tumour antigen. Moreover, ChAd3 presents the advantage to overcome pre-existing immunity and work in conjunction with Ad vaccines based on common human serotypes.

29 ADENOVIRUS WITH BROADENED TROPISM AS AN IMPROVED GENETIC VACCINATION VEHICLE

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In spite of its broad host range, Adenovirus 5 (Ad5) inefficiently transduces a number of clinically relevant tissues and cell types such as adult skeletal muscle and dendritic cells (DC), mostly because of low expression of the Coxsackie-Adenovirus receptor (CAR). We sought to broaden Ad5 tropism by identifying Ad5 fiber knob mutants which recognize novel receptors. We generated a large collection of mutants in the HI loop of the Ad5 fiber knob displayed on the lambda phage. Screening this library identified clones that bind to CAR-negative NIH3T3 cells. Ad5 derivatives incorporating these ligands (Ad5-L) showed a two to three orders of magnitude-enhanced infectivity of the same cells. The same non-native tropism was revealed in other cell types, independent of CAR expression.. We show that Ad5-L derivatives are capable of transducing immature mouse DCs up to 100-fold better than Ad5-wt. Similarly, Ad5-L mutants proved far superior in terms of efficiency of transduction of human primary immature DC. We also demonstrate that Ad5-L mutants are capable of transducing mouse skeletal muscle up to 10-times more efficiently than Ad5-wt. These results spurred us to assess whether mutants could be better suited for use as genetic immunization vectors. Mice were immunized with L-mutant or wt Adenovirus expressing the HIV gag protein. Immune response was monitored through the frequency of IFN-γ secreting spleen cells and antibody titres. We did not detect statistically significant variation in either cell-mediated or humoral immune response between Ad5-L gag and Ad5-wt. Thus we demonstrate that improving the ability of the virus to infect DC or skeletal muscle cells does not affect the efficiency of Ad immunization,. Moreover, Ad5-L mutants have a clear therapeutic potential as vehicles capable of transducing DC, as a route to modulate their function, as well as for the treatment of a wide spectrum of diseases, including cancer. Moreover, increasing the efficiency of muscle transduction can have a remarkable impact on gene therapy protocols.

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30 ROLE OF INFLAMMATORY CYTOKINES ON MODULATION OF CANCER CELL PROLIFERATION

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Inflammatory cytokines have a critical role in modulation of both innate and adaptive immunities in response to foreign antigen. They also play an important role in anti-cancer immunity. For examples, they can promote cell-mediated immunity against cancer cells. With their immunostimulatory effects, these cytokines are being tested for cancer treatment either in the form of DNA vaccine, adjuvant or therapeutic cytokines. Direct effect of these cytokines on cancer cell, however; is still unclear. In this project we investigated if IL-1β and IL-18 can modulate cancer cell proliferation. We employed a simple non-radioactive proliferation (MTT) assay and detection of lactate dehydrogenase (LDH) to test the effect of these recombinant human cytokines on various cancer cell lines including breast cancer cell line (MCF-7), oral carcinoma cell line (KB), and colon cancer cell line (Caco-2). In the presence of serum, IL-1β has no effect on cell number of these cancer cells. However, there was a significant increase of LDH release in MCF-7 treated with this cytokine ($p<0.05$), suggesting a cytotoxic effect on this cell line. Interestingly, as low as 10 pg/mL of IL-18, MCF-7 and KB cell number were augmented ($p<0.05$) with a decrease and no change in LDH release, respectively. In addition, IL-18 treatment in KB cells resulted in a decrease proliferation. Collectively, findings in this *in vitro* study have provided an insight of how cancer cells respond to these cytokines and this could lead to further investigation *in vivo* and a consideration on using cytokine as immunotherapy for cancer treatment.

31 VIRAL CHEMOKINE-ANTIGEN FUSIONS GENERATE PROTECTIVE ANTITUMOR IMMUNITY BY THEIR ABILITY TO TARGET CHEMOKINE RECEPTORS ON APC *IN VIVO*

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Ligands (i.e. chemokines and defensins) for chemokine receptors expressed on APC represent attractive candidates as vaccine carrier, since they can both target and attract APC, while being internalized after binding to the receptor. Fusions of proinflammatory chemokines or defensins with model non-immunogenic tumor or HIV antigens have been shown to efficiently elicit immunity in preclinical models.

Viral chemokines share only partial sequence similarity with human counterparts while retaining the ability to bind to chemokine receptors. Therefore, their use as carrier instead of native chemokines may circumvent the risk of generating autoantibodies against host chemokines. The broad spectrum viral chemokine antagonist vMIP-II genetically fused with a model non-immunogenic tumor antigen (B-cell lymphoma idiotype, Id) generated Id-specific protective immunity at least comparable to that of prototype protein vaccine Ig-KLH. Since viral chemokine administration may lead to host immunization potentially limiting their repeated use, we tested whether pre-existing vMIP-II-specific immunity would affect anti-Id responses to a vMIP-II fusion vaccine. Mice pre-immunized with vMIP-II fused with an irrelevant antigen generated substantial levels of anti-vMIP-II specific antibodies. Nonetheless, these mice could still elicit an anti-Id humoral response, though they showed a lower level of protection from challenge than naive mice.

As vMIP-II has been reported to display an agonistic activity on at least CCR3 and possibly CCR8, to specifically investigate the relative contribution of APC targeting and recruitment in the generation of immunity, we utilized a pair of viral chemokines, agonist vMIP-I and antagonist MC148, which bind to the same receptor inducing and suppressing chemotaxis, respectively. MC148-Id fusions efficiently delivered Id to APC for processing and presentation to antigen-specific T cells *in vitro*. Physical linkage of chemokine and antigen and specific binding to chemokine receptor by the fusion protein were required. Mice immunized with either vMIP-I or MC148 DNA fusion vaccines elicited comparable protection against tumor challenge.

In conclusion, viral chemokines can replace native chemokines as vaccine carrier and their activity depends on their ability to engage chemokine receptors on apcs, while chemotaxis induced by the chemokine moiety in the fusion apparently was not required.

32 CD38 IS A NOVEL MATURATION MARKER FOR HUMAN MONOCYTE-DERIVED DENDRITIC CELLS AND IS INVOLVED IN CD83 AND IL-12 INDUCTION

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Dendritic cells (DCs) maturation is a process characterized by the loss or gain of immunological functions and by the expression of distinctive surface receptors. CD38 is an ectoenzyme that catalyses the synthesis of cyclic-ADP ribose expressed on different lymphoid and myeloid cell populations. CD38 is also a receptor ruling transmembrane signals upon engagement with its counter-receptor CD31, or with agonistic mAbs. The aim of this study was to monitor CD38 expression during the differentiation of human monocytes to immature/mature DCs and to assess the possibility that CD38 plays a functional role during DCs maturation.

CD38 is expressed by monocytes, and down-modulated during the differentiation to immature DCs. Upon maturation CD38 is rapidly re-expressed by DCs, paralleling the reference markers CD83 and CCR7. De novo synthesized CD38 is enzymatically active and its expression is dependent on NF-kB activity. Activation via agonistic anti-CD38 mAb induces up-regulation of CD83 and IL-12. The disruption of CD38 binding with its counter-receptor CD31 markedly inhibits CD83 expression, IL-12 secretion and DCs-mediated induction of alloreactive T lymphocytes proliferation.

In conclusion, our data show that CD38 is not only a novel maturation marker for human DCs, but is also involved in key events taking place during DC maturation. Indeed, CD38 may take part, as a signaling receptor, in the regulation of Th-1 mediated immune responses, by induction of IL-12 production, and in the activation of T lymphocytes, through regulation of CD83 membrane expression.

33 GENERATION OF IMMATURE DC$_S$ CHARACTERIZED BY A TOLEROGENIC PHENOTYPE IN IRF-1 DEFICIENT MICE

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IRF-1 is a transcription factor belonging to the IRF family and playing an important role in the regulation of genes associated with a protective immune response. In this study, we found that the entire dendritic cells (DC) compartment of IRF-1 deficient-mice (IRF-1$^{-/-}$) exhibited an immature phenotype and defective functions in driving T helper cell responses. In particular, the CD8α^+-DCs were found less represented and, as CD8α^- cells, expressing lower levels of activation markers (CD80, CD86, CD40, MHC-II) in IRF-1$^{-/-}$ with respect to wild-type mice. Accordingly, IRF-1$^{-/-}$-DCs expressed low levels of IL-12p40, IL-15, TNF-α, IFN-γ and were defective in stimulating CD8$^+$ and CD4$^+$ T cell proliferation in MLR assays. Intriguingly, IRF-1$^{-/-}$ mice displayed significantly increased number of CD11c^{low} plasmacytoid cells, which showed tolerogenic features. In fact, the plasmacytoid subset CD11c^{low}B220$^+$CD45RA^{high} was greatly augmented and more committed to differentiate into CD8α^+ DCs after virus infection *in vitro*. Accordingly, isolated IRF-1$^{-/-}$ DCs were capable to produce increased levels of IFN type I after viral stimulation in culture as compared to wild-type counterparts. In addition, the recently described tolerogenic CD11c^{low}B220-CD45RB^{high} subset was also enhanced in IRF-1$^{-/-}$ mice. Consistently with a tolerogenic-polarized immune system, we found increased numbers of CD4$^+$CD25$^+$ T regulatory (T_{reg}) cells in all the lymphoid organs of IRF-1$^{-/-}$ mice. Of note, the cytokine analysis revealed a strong expression of IL-10 and IL-4 in IRF-1$^{-/-}$ T_{reg} cells with respect to the CD4$^+$CD25$^-$ T responder cells of same knock-out mice and to the wild-type counterparts. Taken together, these data highlight a new role of IRF-1 in regulating DC differentiation and functions, consisting in allowing their full maturation and, at the same time, in inhibiting the generation of tolerogenic DCs. These findings shed some light on the mechanisms controlling the development of tolerogenic functions and may have practical implications for the identification of novel strategies for preparing DC-based therapeutic vaccines.

34 ICSBP IS CRITICALLY INVOLVED IN THE NORMAL DEVELOPMENT, TRAFFICKING AND ANTIGEN UPTAKE OF DENDRITIC CELLS

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Interferon consensus sequence-binding protein (ICSBP) is a transcription factor belonging to the IRF family, recently shown to play a critical role in the differentiation of defined dendritic cell (DC) populations. Here, we analyzed the role of ICSBP in the development and trafficking of epidermal Langerhans cells (LCs) and dermal DCs and the implications for initiation of a competent immune response. ICSBP$^{-/-}$ mice exhibited a reduced frequency of LCs and a delayed mobility of DCs from skin, which reflected a slower turnover rate in lymph nodes during steady-state conditions. Even under inflammatory changes, ICSBP$^{-/-}$ DCs displayed reduced mobility from skin to lymph nodes and, as a consequence, failed to induce a contact hypersensitivity (CHS) response, suggesting that these DCs were unable to initiate a competent Ag-specific T cell-mediated immunity. Moreover, BM-derived DCs from ICSBP$^{-/-}$ mice exhibited an immature phenotype and a severe reduction of IL-12 expression. These BM-DCs also showed a marked defect in their migratory response to MIP-3alpha, MIP-3beta and 6Ckine, an impaired expression of the chemokine receptors, CCR6 and CCR7, and failed to induce a CHS response to antigen when injected into immunocompetent WT host. Finally, the Ag uptake capacity of DC populations in ICSBP$^{-/-}$ mice was also found to be altered. Together, these results indicate that ICSBP is critically required for the development, trafficking and function of skin DCs, thus playing a critical role in the DC-mediated initiation of T cell immunity. Our data provide insights on DC functional pathways, which may be usefully employed for the design of targeted vaccines for possible clinical applications.

35 MOLECULAR SIGNATURE OF HUMAN DENDRITIC CELLS GENERATED AFTER EXPOSURE OF GM-CSF-TREATED MONOCYTES TO IFN-ALPHA

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We have recently shown that the addition of IFN-α to freshly isolated GM-CSF-treated monocytes results in the rapid generation of highly active DCs. In particular, a 3-day exposure of human monocytes to IFN-α (IFN-DCs) resulted in a marked up-regulation of co-stimulatory molecules, as well as of markers of activation/maturation of DCs, such as CD83, CD25 and CCR7. IFN-DCs also expressed IL-15 and IP-10 (chemokines associated with a Th1 immune response) and MIP-3β. IFN-DCs exhibited a marked chemotactic response to MIP-3β *in vitro* and a strong migratory behavior in SCID mice. In PBL-SCID mice reconstituted with human PBL, the injection of antigen-pulsed IFN-DCs resulted in the induction of a potent primary human antibody response, IFN-γ production and CTL activity (Santini S.M. et al. J. Exp. Med. 191:1777, 2000; Parlato S. et al. Blood 98:3022, 2001; Lapenta C. et al. J. Exp. Med. 198:361, 2003).

In this study, we used the DNA microarray technology to further investigate the molecular mechanisms activated by IFN-α during the DC activation/differentiation process. We have assessed the gene expression profiles in IFN-DCs compared to monocytes treated with GM-CSF alone and to DCs generated with GM-CSF and IL-4 by using Affymetrix GeneChip microarray covering about 22,000 genes. The results showed that a 3-day IFN-α treatment of human monocytes induced a significant modulation of 127 significant genes; in particular, 54 significant genes were up-regulated, while 73 genes were down-regulated with respect to the controls. Of note, we observed a strong induction of the best characterized IFN-inducible genes (2'-5'-oligoadenylate synthetase, Rnase L, pkR) and of transcription factor genes belonging to the IRF family; a considerable up-regulation of a number of molecules involved in the immune response was also detected in IFN-DC. As the IFN-DCs exhibit a remarkable activity in preclinical models suggestive of a possible advantage for their use in the preparation of cancer vaccines, these results can contribute to the identification of novel genes and transcription signatures associated with DC functions important for their use in immunotherapeutic strategies.

36 DENDRITIC CELLS GENERATED AFTER A SHORT-TERM CULTURE OF MONOCYTES WITH IFN-α AND GM-CSF: EVALUATION OF THEIR POTENCY AS CELLULAR ADJUVANTS

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We previously reported that a 3-day culture of human $CD14^+$ monocytes in the presence of GM-CSF and IFN-α leads to the differentiation of activated partially mature dendritic cells, designated IFN-DCs, capable of strongly promoting a T helper cell type 1 response and inducing a potent primary immune response in chimeric SCID mice reconstituted with human PBLs (Santini et al., J. Exp. Med. 191:1777, 2000; Parlato et al., Blood 98:3022, 2001). We also showed that IFN-DCs pulsed with peptides corresponding to Epstein-Barr virus (EBV) CTL epitopes efficiently promote *in vitro* and *in vivo* the expansion of $CD8^+$ T lymphocytes acting as cytotoxic effectors against EBV-transformed cells (Santodonato et al., J. Immunol. 170:5195, 2003). In this study, we further evaluated the potential advantage of using IFN-DCs as cellular adjuvants for cancer immunotherapy. Firstly, we evaluated the ability of IFN-DCs to phagocytose apoptotic tumor cells or tumor cell lysates. In both cases, the IFN-DCs exhibited a significant phagocytic activity, which was similar to that exhibited by immature DCs, generated by cultivation of $CD14^+$ monocytes in the presence of GM-CSF and IL-4. Secondly, we have analyzed the expression levels of the immunoproteasome subunits (PA28, LMP-2, LMP-7, MECL-1) and of TAP molecules in the IFN-DCs as compared to immature or mature DCs. Our results indicate that the levels of these proteins in IFN-DCs are similar to those expressed by mature DCs and up-regulated with respect to those detected in immature DCs, providing further evidence that IFN-DCs exhibit features of mature DCs. Results will be presented concerning the "cross-presentation" ability of IFN-DCs co-cultured with tumor cell-derived material.

37 PERIPHERAL BLOOD MONOCYTES CULTURED IN THE PRESENCE OF GM-CSF ACQUIRE THE PHENOTIPYCAL AND FUNCTIONAL FEATURES OF ACTIVATED DENDRITIC CELLS

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In this study, we report that human peripheral blood monocytes cultured with GM-CSF, in the absence of any other growth factors, acquire a typical DC phenotype within 4-5 days. The expression of CD1a, CD86 and MHC class II overlapped that observed in DCs generated in the presence of GM-CSF and IL-4, whereas higher levels of CD80, CD40 and MHC class I expression as well as of CD14 were found with respect to GM-CSF/IL-4-derived DCs. However, these cells did not acquire the expression of CD71, a molecule specifically up-modulated during monocyte differentiation toward macrophages. Interestingly, differentiation of monocytes into DCs also occurred in the presence of very low GM-CSF concentrations. Upon LPS stimulation of GM-CSF-DCs, a significant down-modulation of CD14 expression was observed, concomitantly with the up-regulation of CD86 expression and the acquisition of the DC maturation markers CD83 and CD25. Immature DCs generated in the presence of GM-CSF alone exhibited a higher allostimulatory activity with respect to GM-CSF/IL-4 DCs. Moreover, upon LPS stimulation, these cells strongly down-modulated their capacity to uptake antigens and acquired the capacity to produce IL-12, although to a lower extent than GM-CSF/IL-4 DCs, and to induce IFN-γ production in mixed cultures with allogenic lymphocytes. However, their already high allostimulatory activity was not further increased upon LPS stimulation. Notably, GM-CSF-generated DCs produced a different pattern of cytokines and chemokines as compared to IL-4-generated cells upon maturation induction. In fact, GM-CSF-generated DCs secreted significantly higher amounts of IL-10 in response to LPS, whereas no TGF-β was detected in both GM-CSF or IL-4-generated cultures. GM-CSF-generated iDCs also released higher levels of both CCL1 and CCL2 as compared to IL-4-generated cells. Moreover, the LPS-stimulated production of these chemokines was found to be higher in DC cultures generated in the presence of GM-CSF.

Experiments are in progress to evaluate the mechanisms underlying the capacity of GM-CSF to drive monocyte differentiation into DCs and to determine whether other cytokines, previously characterised as inducers of DC differentiation/maturation, are involved.

38 CHEMOKINE EXPRESSION AND TRANSENDOTHELIAL MIGRATION PROPERTIES OF HIGHLY ACTIVE DENDRITIC CELLS GENERATED AFTER A 4 HOUR TREATMENT OF MONOCYTES WITH IFN-ALPHA AND GM-CSF

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In a recent study (Eur. J. Immunol. 33:358-367, 2003), we have described a new population of antigen-presenting cells (APC) generated after a surprisingly short time of *in vitro* culture of human monocytes in the presence of IFN-α. In particular, we have demonstrated that freshly isolated $CD2^+$ monocytes can acquire the expression of the dendritic cell (DC) maturation marker CD83 and differentiate into highly active APCs after 4 h of culture in the presence of IFN-α and GM-CSF. Further studies have shown that the these cells (named as "4h-IFN-APC") are able to secrete constitutively high levels of MCP-1 and IP-10 chemokines; of note, the production of MIP-1α, MIP-3β and RANTES chemokines is enhanced after LPS treatment, showing some functional proprieties comparable to those of LPS-treated IL-4 monocyte-derived DCs. Likewise, the 4h-IFN-APC up-regulate CCR7 receptor expression, while no detectable mRNA levels are found in 4h-IL-4-APC as well as in monocytes. Notably, the 4h-IFN-APC exhibit a remarkably high migratory potential in response to chemokines, including MIP-3β. Of interest, the cells migrated in response to chemokine stimuli prove to co-express the both the CD2 and CD83 markers on their cell membrane. At the present time, the large majority of experimental protocols for DC generation require a long time (at least one week) of culture and 2 incubation steps with cytokines and maturation factors in order to obtain active human DCs. The full characterization of this new cell population could contribute to the development of a novel method for preparing highly active DCs from monocytes in a very short time; this may represent an important advantage for the preparation of DC-based cancer vaccines to be used in clinical trials.

39 RECOMBINANT ANTIBODIES WITH MHC-RESTRICTED, PEPTIDE-SPECIFIC, T-CELL RECEPTOR-LIKE SPECIFICITY: NEW TOOLS TO STUDY ANTIGEN PRESENTATION AND EVALUATE ANTI-TUMOR IMMUNITY

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The advent in recent years of the application of tetrameric arrays of class I peptide-MHC complexes now enables us to detect and study rare populations of antigen-specific $CD8^+$ T cells. However, available methods cannot visualize or determine the number and distribution of these TCR ligands on individual cells or detect antigen-presenting cells (APCs) in tissues. Here we describe a new approach that enables to study human class I peptide-MHC ligand-presentation as well as TCR-peptide-MHC interactions. Such studies are facilitated by applying novel tools in the form of peptide-specific, HLA-A2-restricted human recombinant antibodies directed toward a large variety of tumor-associated as well as viral T-cell epitope peptides. Using a large human antibody phage display library, a large panel of recombinant antibodies that are specific for a particular peptide-MHC class I complex in a peptide-dependent, MHC-restricted manner was isolated. These antibodies were used to directly visualize the specific MHC-peptide complex on tumor cells, antigen-presenting cells or virus-infected cells by flow cytometry. They enabled direct quantitation of the number of MHC-peptide complexes as well as in situ detection of the complex on the surface of APCs after naturally occurring active intracellular processing of the cognate antigen. These studies will enable also the development of a new class of targeting molecules to deliver drugs or toxins to tumor or virus-infected cells. Thus, we demonstrate our ability to transform the unique fine specificity but low intrinsic affinity of TCRs into high affinity soluble antibody molecules endowed with a TCR-like specificity toward human tumor or viral epitopes. These molecules may prove to be crucial useful tools for studying MHC class I antigen presentation in health and disease as well as for therapeutic purposes in cancer, infectious diseases, and autoimmune disorders.

40 NEW MODIFICATIONS OF ELISPOT ASSAY FOR MONITORING CTL ACTIVITY IN CANCER VACCINE TRIALS

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The use of IFN-γ ELISPOT assay to evaluate cellular immune responses has gained increasing popularity, especially as a surrogate measure for CTL responses. We successfully applied the assay for monitoring clinical trials. However, the IFN-γ ELISPOT assay does not assess cell-mediated cytotoxicity directly as IFN-γ secretion is not limited to only cytolytic cells. Taking into consideration that the main mechanism of cell-mediated cytotoxicity is the release of cytolytic granules that contain, among others, cytolytic protein Granzyme B (GrB), we developed a modification of GrB ELISPOT assay and applied it to cancer vaccine trials monitoring. Extensive studies demonstrated that the GrB ELISPOT assay is specific, accurately measures the rapid release of GrB, is more sensitive than the 51Cr-release assay, and that it may be successfully applied to measuring CTL precursory frequency in PBMC from cancer patients. Both IFN-γ and GrB ELISPOT assays demonstrated excellent correlations with 51Cr-release assay when PBMC from cancer patents were tested.

The more relevant approach to assess immune responses in cancer patients would be to test the reactivity of T cells against autologous tumor cells. We developed and validated the Autologous Tumor IFN-γ ELISPOT assay. PBMC from Id vaccinated lymphoma patients as effectors and autologous B cell lymphoma tumor cells as targets were used. We demonstrated that this assay could be used to reliably and reproducibly determine the tumor-reactive T cell frequency in the PBMC of these patients.

The data presented show that our modifications of the ELISPOT assay may be successfully applied to detection of low frequency, tumor-specific CTL and their specific effector functions in variety of cancer vaccine trials.

41 NKG2D$^+$ T CELLS ARE PRESENT IN TUMOR INFILTRATING LYMPHOCYTES (TILS) OF MELANOMA PATIENTS AND EXERT IN-VITRO ANTI-TUMOR ACTIVITY

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NKG2D is an activatory receptor expressed by NK and T cells that can mediate an anti-tumor immune response. The goal of this study was to evaluate whether NKG2D$^+$ T cells are detectable in tumor infiltrating lymphocytes from melanoma patients and whether these T cells exerted anti-tumor reactivity. We have found that NKG2D$^+$ T cells, as shown by immunohistochemical analysis, commonly infiltrated melanoma lesions. Moreover, tumor samples expressed heterogeneously the NKG2D ligands: ULBP2 in 10/10, MICA and ULBP1 in 4 and 3 out of 10 samples, respectively, MICB only in 1/10 melanoma primary lesion, whereas ULBP3 was never detected. These results were confirmed by immunofluorescence analysis performed on either freshly isolated and short term in-vitro cultured TILs or on tumor cell lines derived from some of these patients. NKG2D$^+$ T cells isolated from TILs were shown to mediate in-vitro specific recognition of autologous or allogeneic tumor cell lines, as shown by cytokine release after co-incubation with tumor cells, and this activity was exerted concomitantly by TCR and NKG2D. Similar reactivity was observed by CD8$^+$ or CD4$^+$ T cell clones derived from TILs of one melanoma patient. Furthermore, we found that NKG2D can mediate anti-tumor recognition by T lymphocytes specific for a defined TAA. In fact, both TCR and NKG2D could mediate recognition of autologous melanoma cells by gp100-specific T lymphocytes isolated from a tumor invaded lymph node (LN) or PBMCs and stimulated in-vitro with the same peptide. On the contrary, one NKG2D$^+$ CD8$^+$ T cell clone isolated from invaded LNs of the same patient exerted anti-tumor activity independently from NKG2D. Moreover, these results were confirmed by the analysis of one NKG2D$^+$ CD8$^+$ T cell clone directed to a different gp100-derived epitope. Interestingly, recognition of autologous tumor cell lines by a CD8$^+$ T cell clone specific for the mutated melanoma associated antigen, β-catenin, was achieved by concomitantly engagement of TCR and NKG2D. Further investigation need to be performed in a larger panel of melanoma patients at different stage of the disease to confirm our data. Taken together, these results show that NKG2D$^+$ T cells, either isolated from melanoma lesions or from in-vitro stimulated PBMCs, represent relevant subpopulations for the anti-tumor immune response. Conversely, NKG2D-dependent tumor recognition occurs heterogeneously among in-vitro selected T cell clones.

42 CHARACTERIZATION OF TUMOR-ASSOCIATED T CELLS: BENIGN VERSUS MALIGNANT EFFUSIONS

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Purpose: While naive and central memory T cells circulate between peripheral blood and lymph nodes, only memory effector T cells acquire certain surface molecules that enable them to travel to peripheral tissues and exert their effector function. Therefore, an active immunotherapy has to guarantee the generation of T cell subtypes that are capable of homing into the tumor tissue. We investigated whether a defective homing of effector T cells to the tumor might contribute to tumor escape from immunosurveillance.

Experimental design: We analyzed the expression of a broad range of adhesion molecules and chemokine receptors (CD62L, CD56, CCR4, CCR5, CCR7, CXCR3, CLA, integrin $\alpha 4\beta 7$) on tumor-associated lymphocytes (TAL) in effusions and peripheral blood lymphocytes (PBL) of patients with malignant ascites (N=11) or malignant pleural effusion (N=16). A TAL / PBL ratio was calculated as an indicator for homing of lymphocytes into the effusions and was compared to patients with non-malignant ascites (N=17).

Results Patients with malignancies show an increased enrichment of T cells expressing the phenotype of "naïve" ($CD62L^+$, $CD45RA^+$, $CCR7^+$), "central memory" ($CD45RA^-$ $CCR7^+$), and type 2-polarized ($CCR4^+$) T cells within their effusions. In contrast, homing of "effector"-type ($CD45RA^-CCR7^-$ or $CD45RA^+CCR7^-$) and presumably type 1-polarized T cells ($CCR5^+$) from the peripheral blood to the tumor site is deficient. In addition, cancer patients showed a diminished homing of NK cells and potentially cytotoxic $CD56^+$ T cells into their effusion.

Conclusions: Here we show for the first time that patients with malignant effusions show a deficient homing of T cells expressing the phenotype of type-1-polarized effector T cells from the peripheral blood to the tumor site. This mechanism is likely to contribute to the escape of tumor cells from immunosurveillance. Therefore, clinical immunization studies should not only focus on the generation of antigen-specific T cells but should also examine the potential of these cells to travel into the cancer tissue.

43 CANCER VACCINE DERIVED FROM PLANT VIRUSES: CHIMERIC POTATO VIRUS X PARTICLES EXPRESSING A MELANOMA ASSOCIATED T CELL EPITOPE

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The potential of epitopes represented by synthetic peptides for the development of cancer vaccines is under investigation. Although impressive progress was made the efficacy of peptides in the induction of an immune response is limited mainly because of the short half-life in the serum. Several delivery systems have been devised to circumvent this limitation. A possibility is to administer modified viral particles displaying epitopes on their surface. Plant viruses are currently being exploited for this kind of application and their employment is of particular interest because they are harmless for human health. Our work is focused on the use of Potato Virus X (PVX) as a vaccine delivery system. We have previously demonstrated that the mucosal delivery of Potato Virus X (PVX) chimeric particles (CVPs) expressing an HIV-1 derived epitope is able to induce strong antibody responses in several animal models, without the need of adjuvants (Marusic C. et al., J.Virol. 75: 8434-8439, 2001). We are presently evaluating the capability of PVX CVPs to elicit specific cytotoxic T cell responses by entering the antigen presenting cells compartments resulting in MHC class I presentation despite the fact they are not animal pathogens. To this aim we have constructed PVX CVPs expressing an epitope derived from the melanoma associated protein Melan A/MART-1 (Melanoma Antigen Recognized by T cells -1). This epitope is a good vaccine candidate being expressed in more than 80% of metastatic tumors. A modified viral expression vector, derived from pPVX201 has been constructed to insert as a coat protein fusion an HLA-A2 restricted epitope corresponding to aminoacid positions 27-35 of MART-1 (AAGIGILTV). The recombinant vector was used to infect Nicotiana benthamiana plants and was able to generate virus particles systemically infecting plants and producing typical symptoms. The presence of chimeric coat protein was confirmed by RT-PCR and sequence analysis. Virion stability through generation was verified by repeated infection cycles with infected plant extracts, followed by molecular analysis.

Immunological properties of chimeric virus particles purified from plant tissues are currently under scrutiny by *in vitro* assays to assess their ability to stimulate $CD8^+$ T-cell lines expressing MART-1 epitope specific T cell receptors.

44 GENE EXPRESSION PROFILING OF TUMOR BIOPSIES TO IDENTIFY PREDICTORS OF CLINICAL OUTCOME IN PATIENTS WITH MALIGNANT MELANOMA

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Despite significant efforts to identify independent predictors of melanoma outcome, no accepted histopathological, molecular or immunohistochemical markers define subsets of this neoplasm. The important genetic changes responsible for melanoma progression are poorly characterized, and the heterogeneity of the clinical course of the disease is unexplained. As a consequence, melanoma continues to be an unpredictable cancer. In search for predictors of clinical outcome, we recently observed that the ability of tumor cells to grow *in vitro* and to establish a cell line seemed to be strictly correlated to the prognosis in patients with stage III and IV cutaneous melanoma. A total of 59 melanoma tissue samples were collected from patients undergoing clinical evaluation either as a part of the diagnostic work-up or in order to select individuals for peptide vaccination trials. Cell lines with morphologic, immunophenotypic and antigenic characteristics of melanoma cells were successfully established in 22 out of 59 patients. The median survival for patients with or without established cell line was 8.3 and 49.5 months, respectively (Kaplan-Meier test, $p = 0.0001$ in a two-tail Logrank test). This result seems to indicate a strict association between the ability of melanoma cells to grow *in vitro* and their potential to proceed towards an adverse clinical course.

Aim of the present study was to analyze the transcriptional profiles of melanoma biopsies from which cell lines could or could not be established *in vitro*. Anti-sense RNAs (aRNA) were prepared from the 2 groups of samples and hybridized to cDNA microarrays with 17,500 oligonucleotidic probes. Gene expression levels of each specimen were compared to an RNA reference derived from pooled peripheral blood mononuclear cells of six donors.

Supervised and unsupervised algorithms were used to analyze the expression profiling data and to identify subsets of putative prognosis predictors. A cross-validated penalized Cox model was used to highlight the correlation between transcriptional profiles and survival data. Experiments are ongoing to confirm differentially expressed genes and to identify candidates or pathological pathways that might lead to the definition of a molecular profile for highly aggressive melanomas.

45 IDENTIFICATION OF CYTOMEGALOVIRUS T CELL EPITOPES USING THE ITOPIA™ EPITOPE DISCOVERY SYSTEM

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Stimulation of the T cell response to specific antigens is an emerging technique used in vaccine discovery. The iTopia™ Epitope Discovery System from Beckman Coulter allows vaccine developers working on T cell mediated vaccines to map and characterize direct binding of epitopes to MHC complexes. We used the iTopia™ Epitope Discovery System to analyze the CMVpp65 matrix protein and identify immunogenic epitopes from this protein across 9 MHC alleles.

The iTopia Epitope Discovery System, which allows the rapid *in vitro* identification of peptides binding to a given Class I MHC molecule, analyzes the direct binding, affinity and off-rates for the possible peptide/allele combinations of a selected protein. This allows a systematic ranking of candidate epitopes for subsequent functional studies and speeds the process of identifying immunogenic epitopes for disease monitoring and vaccine development. The knowledge of the binding peptides also allows subsequent synthesis of iTAg™ MHC Tetramers which are an important tool to visualize and quantify antigen-specific CTL response, in this case to CMV reactivation.

The iTopia Epitope Discovery System was used to screen the entire CMV pp65 matrix protein, the major immunogenic protein of CMV, by analyzing all overlapping nonamers (553 peptides) across 9 different HLA-A/B alleles: HLA*A0101, HLA*A0201, HLA*A0301, HLA*A1101, HLA*A2402, HLA*B0702, HLA*B0801, HLA*B1501, HLA*B2701. After an initial step of determining the overall binding of each peptide, medium and high binders were analyzed for binding affinity and peptide dissociation rates. The results, or iScores, of multi-parametric analysis based on the affinity and dissociation values for each peptide permitted us to select the potential candidate epitopes for each allele.

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