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International Meeting

**Cytokines as natural adjuvants:
perspectives for vaccine development**

Istituto Superiore di Sanità
Rome, 22-24 April 2002

ABSTRACT BOOK

Edited by
Maria Ferrantini, Franca Moretti and Filippo Belardelli

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International Meeting. Cytokines as natural adjuvants: perspectives for vaccine development. Istituto Superiore di Sanità. Rome, 22-24 April 2002. Abstract book.

Edited by Maria Ferrantini, Franca Moretti and Filippo Belardelli
2002, vi, 94 p. ISTISAN Congressi 02/C2

The Meeting is organized with the objective of dedicating special attention to the recent developments of research on the use of cytokines as natural adjuvants for the development of vaccines directed against infectious and neoplastic diseases. Cytokines are the major mediators of host defenses against infectious agents and tumors, in that they regulate the communication between cells of the immune system. In particular, certain cytokines act as a link between the innate and adaptive immune responses, promoting the development of immunity against pathogens and tumors. Recent progress in the field of immune regulation has led to the concept of using certain cytokines as natural adjuvants for human vaccines. The major aims of the Meeting are: i) to review the most recent findings of the basic and applied research on the ability of cytokines to induce a protective immunity; ii) to discuss the perspectives of the use of cytokines for the development of vaccines effective in preventing or treating human infectious diseases and malignancies.

Key words: Cytokines, Adjuvants, Vaccines

Istituto Superiore di Sanità

Convegno internazionale. Citochine come adiuvanti naturali: prospettive per lo sviluppo di vaccini. Istituto Superiore di Sanità. Roma, 22-24 aprile 2002. Riassunti.

A cura di Maria Ferrantini, Franca Moretti e Filippo Belardelli
2002, vi, 94 p. ISTISAN Congressi 02/C2 (in English)

Il Convegno è realizzato con l'obiettivo di dedicare una particolare attenzione ai recenti sviluppi della ricerca sull'uso di citochine come adiuvanti naturali per lo sviluppo di vaccini diretti contro malattie infettive e neoplastiche. Le citochine sono i principali mediatori della risposta difensiva contro agenti infettivi e tumori, in quanto regolano le interazioni tra le cellule del sistema immunitario. In particolare, alcune citochine agiscono come ponte tra la risposta immune innata e acquisita, promuovendo lo sviluppo dell'immunità contro patogeni e tumori. I progressi recenti nel campo della regolazione della risposta immune hanno fornito un supporto scientifico al concetto dell'utilizzo di alcune citochine come adiuvanti naturali per la vaccinazione nell'uomo. I principali obiettivi del Convegno sono: a) offrire una rassegna delle più recenti scoperte della ricerca di base e applicata sulla capacità delle citochine di indurre una immunità protettiva; b) discutere le prospettive dell'uso di citochine per lo sviluppo di vaccini efficaci nella prevenzione o il trattamento di malattie infettive e neoplasie umane.

Parole chiave: Citochine, Adiuvanti, Vaccini

Meeting organized by: Istituto Superiore di Sanità in collaboration with the Italian Association of Immunology, Clinical Immunology and Allergology.

Scientific Committee

Filippo Belardelli, Maria Ferrantini, Alberto Mantovani, Giorgio Parmiani

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PROGRAM

Monday, 22 April 2002

- 8.30 Registration
9.30 Welcome and opening address
E. Garaci

Session I

DENDRITIC CELLS AS KEY PLAYERS IN THE IMMUNE RESPONSE

Chairpersons: L. Moretta - V. Barnaba

- 9.40 *Memory stem cells*
A. Lanzavecchia
- 10.10 *Human plasmacytoid dendritic cells in innate and adaptive immunity*
M. Gillet
- 10.40 Break
- 11.10 *The major type I interferon producing cells in the mouse are immature antigen-presenting cells exhibiting plasmacytoid morphology*
G. Trinchieri
- 11.40 *Biology of mouse dendritic cells and vaccine development: the IL-2 mediates the adjuvant effect of dendritic cells*
P. Ricciardi-Castagnoli
- 12.10 *Are dendritic cells a barrier to mucosal immunisation?*
G. MacPherson
- 12.40 *Overlapping but distinct effects of IL-23 and IL-12 on murine dendritic cells*
M.L. Belladonna
- 13.00 Lunch
- 13.30 **Poster session**

Session II

CYTOKINES AND TH1/TH2 POLARIZATION OF IMMUNE RESPONSE

Chairpersons: G. Doria - A. Santoni

- 14.30 *Chemokines in innate and adaptive immunity*
A. Mantovani

- 15.00 *Regulation of immune responses by type I interferons*
D.F. Tough
- 15.30 *IL-12, a key regulatory cytokine in Th1-mediated responses*
L. Adorini
- 16.00 Break
- 16.30 *Reciprocal activating interaction between natural killer cells and dendritic cells*
F. Gerosa
- 16.50 *Role of cytokines in mediating dendritic cells and epithelial cells cross-talk*
M. Rescigno
- 17.10 *Antibody-triggering of the mannose receptor elicits maturation of myeloid dendritic cells secreting IL-10, no IL-12 and a distinct chemokine profile*
M. Chieppa
- 17.30 *Role of CTLA-4 in Th subset polarization*
C. Pioli

Tuesday, 23 April 2002

Session III

ADJUVANTS AND CYTOKINES IN INFECTIOUS DISEASES

Chairpersons: A. Cassone - B. Ensoli

- 9.00 *Cytokines and Th1 vs Th2 response in humans*
S. Romagnani
- 9.30 *A push-pull approach to maximize vaccine efficacy: augmenting help and abrogating suppression with cytokines, costimulatory molecules, and their inhibitors*
J.A. Berzofsky
- 10.00 *Interleukin-2 enhances HIV-specific immune responses only when used concomitantly with the antigen*
F. Lori
- 10.20 *Flt3 ligand and GM-CSF adjuvants for Hepatitis B vaccination of healthy adults*
T.G. Evans
- 10.50 *Pertussis toxin induces maturation of human monocyte-derived DC and promotes IL-12 dependent Th1 polarization of naive cord blood T cells in synergic association with LPS*
C.M. Ausiello

- 11.10 Break
- 11.40 *New adjuvants*
R. Rappuoli
- 12.10 *Type I IFN as a natural adjuvant necessary and sufficient for a protective immune response: lessons from the influenza model*
E. Proietti
- 12.30 *Transgenic Plasmodium knowlesi produces bioactive interferon gamma*
H. Ozwara
- 12.50 *Efficient delivery of T-cell epitopes to APC by use of MHC class II-specific Troj-bodies*
K. Western
- 13.10 Lunch
- 13.30 **Poster session**

Session IV
CYTOKINES AND CANCER VACCINES:
WHAT HAVE WE LEARNED FROM ANIMAL MODELS?

Chairpersons: E. Bonmassar - G. Rasi

- 14.30 *Cytokines and DNA vaccines in the prevention of Her-2/neu carcinogenesis in BALB/c mice*
G. Forni
- 15.00 *Cytokines and molecularly defined adjuvants can either enhance or restrain the efficacy of recombinant cancer vaccines*
V. Bronte
- 15.30 *In vivo targeting of dendritic cells using cellular vaccines*
M.P. Colombo
- 16.00 Break
- 16.30 *Dendritic cells from bench to bedside*
G. J. Adema
- 17.00 *Enhancing Anti-tumor Immunity with Autologous Stem Cell Transplantation*
I. Borrello
- 17.30 *IFN-gamma-independent responses induced by mammary adenocarcinoma cells engineered with IL-15 and IL-12 gene in syngeneic mice*
S. Ferrini

Wednesday, 24 April 2002

Session V

**CLINICAL USE OF CYTOKINES IN VACCINE DEVELOPMENT:
STATE OF THE ART AND NEW PERSPECTIVES**

Chairpersons: G. D'agnolo - R. Foà

- 9.00 *Cytokines in cancer vaccines*
 G. Parmiani
- 9.30 *High-dose interferon (HDI) - Cornerstone of present adjuvant therapy
 for melanoma, and a platform for future progress*
 J.M. Kirkwood
- 10.00 *Perspectives in the use of type I IFN and dendritic cells for the development
 of human vaccines*
 F. Belardelli
- 10.30 *Cancer vaccination with peptides and granulocyte macrophage stimulating factor
 (GM-CSF)*
 E. Jäger
- 11.00 Break
- 11.30 *Cross-priming of tumor-specific T cells in vitro and in melanoma patients
 using autologous dendritic cells as natural adjuvant*
 J.-P. Abastado
- 12.00 *Immunogenic role of the apoptosis inhibitor protein survivin
 in colo-rectal cancer patients*
 C. Casati
- 12.20 *Ex vivo priming and expansion of donor-derived long-term
 anti-leukemia CTL lines*
 D. Montagna
- 12.40 End of the meeting

Session I
Dendritic cells as key players
in the immune response

Chairpersons
Lorenzo Moretta - Vincenzo Barnaba

MEMORY STEM CELLS

Antonio Lanzavecchia

Institute for Research in Biomedicine, Bellinzona, Switzerland

Immunological memory lasts for a lifetime. It confers to primed individuals a certain level of immediate protection as well as the capacity to mount secondary immune responses. These aspects of immunological memory have a distinct cellular basis. Protective memory is mediated in peripheral tissues by “effector-memory” T cells and by antibodies, while reactive memory is mediated by “central- memory” T cells and memory B cells that respond to antigenic stimulation in secondary lymphoid organs. We are studying the mechanisms that control the generation of different types of memory cells and their maintenance in the absence of antigen. We found that lymphocyte activation by antigen is a progressive and stochastic process that leads to the generation of both effector cells and intermediates. The latter represent the central memory component of the immune response and have stem cell properties. In the absence of antigen central memory T and B lymphocytes continuously proliferate in response to signals delivered by cytokines, pathogens or T cells and, while self renewing, spill out terminally differentiated effector T cells and plasma cells. This mechanism is required to continuously replenish effector T cells and plasma cells that turn over or are eliminated, thus maintaining constant levels of protective memory. I will discuss the implications of the “stem cell model” of immunological memory for vaccination, immune reconstitution and autoimmunity.

HUMAN PLASMACYTOID DENDRITIC CELLS IN INNATE AND ADAPTIVE IMMUNITY

Michel Gilliet^{1,2}, Norimitsu Kadowaki², Vassili Soumelis² and Yong-Jun Liu²

¹*Department of Dermatology, Zurich University Hospital, Zurich, Switzerland*

²*DNAX Research Institute, Palo Alto, CA, USA*

Plasmacytoid pre-dendritic cells (pre-DC2) or type 1 IFN-producing cells (IPC) represent a unique cell type in the hematopoietic system, expressing a distinct set of pattern recognition receptors (TLR7 and TLR9). In response to viral or bacterial infection pre-DC2 rapidly produce large amounts of type 1 interferon and differentiate into dendritic cells with the ability to polarize naive T cells into IFN-gamma-producing cells. By contrast, in the presence of IL-3 and CD40-ligand pre-DC2 differentiate into mature dendritic cells (DC2) with the capacity to drive TH2 polarization. We analyzed the role of CD40-ligand activated DC2 in priming CD8 T cells. Naive CD8 T cells primed *in vitro* with allogeneic DC2 differentiated into a population of IL-10-producing CD8 T cells, which were anergic and non-cytolytic. In direct coculture experiments we demonstrated that these cells are CD8 T regulatory cells capable of inhibiting primary T cell responses in a dose-dependent manner through the production of IL-10 but not TGF-beta. Transwell experiments indicated that suppression of bystander T cells activated by a third party APC could occur, provided that IL-10 producing CD8 T regulatory cells were restimulated in an antigen-specific manner. In addition, we showed that the induction of IL-10 producing CD8 T regulatory cells was an intrinsic property of CD40-ligand activated DC2, mediated by mature cells, regardless of the kinetics of activation and the lack of relevant IL-12 production. These data suggest a role of DC2 in immunological tolerance and provides a cellular basis for the phenomenon of CD8 T cell-mediated immunosuppression.

THE MAJOR TYPE I INTERFERON PRODUCING CELLS IN THE MOUSE ARE IMMATURE ANTIGEN- PRESENTING CELLS EXHIBITING PLASMACYTOID MORPHOLOGY

Giorgio Trinchieri

*Schering-Plough Research Institute, Laboratory of Immunological Research, Dardilly,
France*

Mouse IFN- α producing cells (MIPC) are a new and unique subset of immature antigen-presenting cells secreting high amounts of IFN- α upon *in vitro* stimulation with influenza virus. Freshly isolated MIPC had a plasmacytoid morphology, stained with an antibody against Ly6G/C (Gr-1) and expressed Ly6C, B220, low levels of CD11c and CD4 but neither CD8 α nor CD11b unlike previously described mouse DC subsets. In response to influenza virus and CpG oligonucleotides, but not to bacterial products, MIPC also produce IL-12. *In vitro*, MIPC underwent rapid apoptosis unless rescued by virus, IFN- α or CpG ODN stimulation that enhanced their survival and endowed them with T cell stimulatory activity. *In vivo* depletion of Ly6G/C cells totally abrogated the production of type I IFN in the serum of mice infected with cytomegalovirus, thus demonstrating that this was the main population, *in vivo*, producing type I IFN in response to this virus.

BIOLOGY OF MOUSE DENDRITIC CELLS AND VACCINE DEVELOPMENT: THE IL-2 MEDIATES THE ADJUVANT EFFECT OF DENDRITIC CELLS

Francesca Granucci and Paola Ricciardi-Castagnoli

Department of Biotechnology and Bioscience, University of Milan "Bicocca", Milan, Italy

Dendritic cells (DCs) are strong activators of primary T cell responses. They acquire this ability upon the encounter of maturation stimuli represented in vivo by intact microorganisms or microorganism components like LPS and viral and bacterial DNA. In order to identify genes involved in DC maturation, a kinetic study of DC gene expression following activation with Gram-negative bacteria was performed using microarrays representing 11000 genes and ESTs. The growth factor dependent D1 cells were used as source of DCs. A large number of transcripts (2951) differentially expressed during DC maturation were identified. These sequences mostly encoded enzymes, transcription factors, signal transduction molecules and proteins involved in cytoskeleton rearrangements and inflammatory responses. Unexpectedly, the interleukin (IL)-2 transcript was transiently upregulated at early time points following bacterial encounter. By comparing the ability of early activated wild type and IL-2^{-/-} DCs to stimulate alloreactive T cells, we have shown that IL-2 represents an additional key molecule conferring unique T cell stimulatory capacity to DCs.

ARE DENDRITIC CELLS A BARRIER TO MUCOSAL IMMUNISATION?

Gordon MacPherson, Fang-Ping Huang and Emma Turnbull
Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

In contrast to the immunity resulting from natural infection many current vaccines used for mucosal immunisation are weak or only partially effective. Dendritic cells (DC) acquire antigens in the intestinal wall and transport them within Peyer's patches and to mesenteric nodes for induction of immune responses. Contrary to some current dogma, we have shown that in the rat DC migration from the intestine to mesenteric nodes is constitutive in absence of any inflammation or 'danger'. We have also shown that one subpopulation of these migrating DC transports apoptotic enterocytes to the T cell areas of mesenteric nodes. These DC are however weak antigen-presenting cells (APC) and we suggest are involved in the induction and maintenance of self tolerance. The other migrating DC population (strong APC) is excluded from the T cell area under steady-state conditions. Following a pro-inflammatory stimulus (i.v. LPS) the strong APC DC population migrates into the T cell area of the node.

We suggest that to induce an optimal response to mucosal vaccines, additional, non-antigenic stimuli may be needed to convert DC behaviour from tolerogenic to immunogenic.

OVERLAPPING BUT DISTINCT EFFECTS OF IL-23 AND IL-12 ON MURINE DENDRITIC CELLS

Maria Laura Belladonna, Roberta Bianchi, Carmine Vacca, Francesca Fallarino, Ciriana Orabona, Maria Cristina Fioretti, Ursula Grohmann, Paolo Puccetti
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IL-23 is a recently discovered heterodimeric cytokine that shares biological properties with proinflammatory cytokines. The biologically active heterodimer consists of p19 and the p40 subunit of IL-12. IL-23 has been shown to possess biological activities on T cells that are similar as well distinct from IL-12. We have constructed single chain IL-23 and IL-12 fusion proteins (IL-23-Ig and IL-12-Ig) and have compared the two recombinant proteins for effects on murine dendritic cells (DC). Here we show that the IL-23-Ig can bind a significant proportion of splenic DC of both the CD8 α^- and CD8 α^+ subtypes. Furthermore, IL-23- and IL-12-Ig exert biological activities on DC that are only in part overlapping. While both proteins induce IL-12 production from DC, only the IL-23-Ig can act directly on CD8 α^+ DC to promote immunogenic presentation of an otherwise tolerogenic tumor peptide. In addition, the effects of IL-23-Ig did not appear to require IL-12 receptor β 2 subunit and to be mediated by production of IL-12. These data may establish IL-23 as a novel cytokine with major effects on APC and be instrumental in the design of optimal vaccination strategies in tumor immunotherapy.

Session II
Cytokines and Th1/Th2 polarization
of immune response

Chairpersons
Gino Doria - Angela Santoni

CHEMOKINES IN INNATE AND ADAPTIVE IMMUNITY

Alberto Mantovani

*Department of Immunology and Cell Biology, Institute for Pharmacological Research
"Mario Negri", Milan, and Istituto of Pathology, University of Milan, Milan, Italy*

Chemokines are a complex superfamily of mediators able to recruit diverse leukocytes. The chemokine system is regulated at the level of agonist availability, as illustrated by macrophage derived chemokine/CCL22, a molecule which is expressed constitutively in certain tissues, it is induced by master cytokines of polarized responses and is processed by CD26. In addition, the system is also regulated at the level of the receptors, by changing levels of expression and coupling. In the presence of IL-10 and strong proinflammatory signals, certain chemokine receptors are uncoupled and act as a sink (decoy) for the agonists. Leukocyte recruitment in tumor tissues is guided by chemokines, the prototype of which is MCP-1/CCL2. Chemokines are also used to subvert and divert effective antiviral/antitumor immunity by amplifying polarized type II responses: this concept was demonstrated for the three chemokines encoded by KSV/HHV8. Chemokines play a key role in guiding dendritic cell trafficking. Available information suggests that they are important for the localization of these cells in tumors and that they may be useful to activate antitumor immunity.

REGULATION OF IMMUNE RESPONSES BY TYPE I INTERFERONS

David F. Tough¹, Giovanna Schiavoni², Fabrizio Mattei², Maria Montoya¹,
Giuseppina D'Agostino², Ion Gresser³, Persephone Borrow¹, Filippo Belardelli²,
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Type I interferons (IFN-I) are expressed rapidly following infection, and play a key role in the early inhibition of virus replication. The initial effects of IFN-I are mediated through both induction of genes that “interfere” with virus replication and activation of innate immune cells (i.e., NK cells and macrophages) that can recognise and kill infected cells. However, since expression of IFN-I coincides temporally with the initiation of the adaptive immune response, it is possible that these cytokines also play a role in immune-mediated defence against infection, perhaps acting as natural adjuvants. We have investigated the effects on the immune response of either inducing expression of IFN-I in the host or administering IFN-I at the time of immunisation. We found that IFN-I enhanced both T cell and antibody responses to soluble proteins; antibodies elicited following immunisation and treatment with IFN-I included all subclasses of IgG. Furthermore, administration of IFN-I promoted the development of long-lived antibody production and immunological memory after a single injection of antigen. IFN-I were able to enhance the immune response *in vivo* when dendritic cells (DCs) were the only cells able to respond directly to these cytokines, implicating DCs as key target cells for the action of IFN-I. Consistent with this idea, IFN-I were shown to induce phenotypic and functional activation of DCs, and to modulate the cytokines expressed by DCs. Interestingly, treatment with IFN-I resulted in increased expression of both IL-15 and the IL-15R alpha-chain by DCs, while exposure of DCs to IL-15 stimulated their phenotypic and functional activation. The results identify IFN-I and IL-15 as key cytokines linking innate and adaptive immunity.

IL-12, A KEY REGULATORY CYTOKINE IN TH1-MEDIATED RESPONSES

Luciano Adorini
BioXell, Milan, Italy

IL-12, a heterodimeric cytokine produced by different types of antigen-presenting cells and in particular by dendritic cells, exerts a decisive role in Th1 cell development and is involved in the pathogenesis of Th1-mediated autoimmune diseases. We have previously shown that IL-12 administration in nonobese diabetic (NOD) mice induces acceleration of type 1 diabetes (T1D) associated with enhanced IFN-gamma-producing Th1 cells in the pancreas. Since IL-12 is a powerful IFN-gamma inducer, we asked if IFN-gamma is simply a marker of Th1 cells or is required for T1D acceleration. We have analyzed T1D pathogenesis in IFN-gamma-deficient and wild-type NOD mice following IL-12 administration. Intriguingly, IL-12-mediated acceleration of T1D was found to be IFN-gamma-independent. In addition, our results indicate that IL-12 administration induces the expansion of a myeloid-type DC population that could play a role in the IFN-gamma-independent T1D acceleration. IL-12 administration can also reveal pathogenic T cells in genetically resistant mice. NOD and NOD-DR alpha transgenic mice develop T1D, whereas NOD-E alpha transgenics are protected. IL-12 administration induces rapid T1D onset in NOD-DR alpha but fails to provoke insulinitis and T1D in NOD-E alpha transgenic mice. Nevertheless, T cells from IL-12-treated NOD-E alpha transgenic mice secrete IFN-gamma and transfer T1D to NOD-SCID and NOD-E alpha-SCID recipients, demonstrating the unveiling of peripheral diabetogenic Th1 cells in the protected mice. These results show the multi-faceted role of IL-12 in the pathogenesis of Th1-mediated autoimmune diseases and raise a note of caution about its use as vaccine adjuvant.

RECIPROCAL ACTIVATING INTERACTION BETWEEN NATURAL KILLER CELLS AND DENDRITIC CELLS

Franca Gerosa¹, Barbara Baldani-Guerra¹, Carla Nisii¹, Viviana Marchesini¹,
Giuseppe Carra¹ and Giorgio Trinchieri²

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²*Schering-Plough Research Institute, Laboratory for Immunological Research, Dardilly, France*

We analyzed the interaction between human peripheral blood natural killer (NK) cells and monocyte-derived immature dendritic cells (DC). Fresh NK cells were activated, as indicated by the induced expression of the CD69 antigen, and their cytolytic activity was strongly augmented by contact with LPS-treated mature DC or with immature DC in the presence of the maturation stimuli LPS, *Mycobacterium tuberculosis* or IFN- α . Reciprocally, fresh NK cells cultured with immature DC in the presence of the maturation stimuli strongly enhanced DC maturation and IL-12 production. IL-2-activated NK cells directly induced maturation of DC and enhanced their ability to stimulate allogeneic naïve CD4⁺ T cells. These effects of NK cells were cell contact dependent, although the secretion of IFN- γ and TNF also contributed to DC maturation. Within peripheral blood lymphocytes the reciprocal activating interaction with DC was restricted to NK cells, because the other lymphocyte subsets neither were induced to express CD69 nor induced maturation in contact with DC. These data demonstrated for the first time a bi-directional cross-talk between NK cells and DC in which NK cells activated by IL-2 or by mature DC in turn induce DC maturation.

ROLE OF CYTOKINES IN MEDIATING DENDRITIC CELLS AND EPITHELIAL CELLS CROSS-TALK

Gianluca Rotta¹, Marisa Vulcano², Silvano Sozzani² and Maria Rescigno¹

¹*Department of Experimental Oncology, European Institute of Oncology, Milan, Italy*

²*Istituto for Pharmacological Research “Mario Negri”, Milan, Italy*

The intestinal mucosa is covered by a single layer of epithelial cells that are inaccessible to pathogens due to the presence of a brush border on the luminal cell surface and of tight junctions between cells. Entry of pathogens occurs mainly through specialized epithelial cells, called M cells, which are located in Peyer's patches (PP), but we have recently described a new mechanism for bacterial entry which is mediated by dendritic cells (DC). Dendritic cells have the capacity to open the tight junctions and to sample directly antigens across epithelia, both *in vitro* and *in vivo*. Uptake of bacteria across the intestinal lumen is not restricted to pathogenic bacteria because also a laboratory strain of *E. coli* is internalized by DC. DC express proteins involved in the formation of tight junctions, such as occludin, claudin 1 and Zonula occludens 1, thus preserving the integrity of the epithelial barrier. We have analyzed the role of cytokines in mediating DC-epithelial cells cross-talk in terms of DC recruitment from the blood and subsequent modulation of tight junction proteins. Epithelial cells have been tested for their capacity to release proinflammatory cytokines such as TNF- α , IL1 β and of chemokines such as IL8, MIP1 α , MIP3 α and MIP3 β after encounter with *Salmonella typhimurium*. Epithelial cells have been incubated with several *Salmonella* strains deficient in different pathways of pathogenicity and invasiveness. The capacity of cytokines produced by epithelial cells to activate DC is also discussed.

ANTIBODY-TRIGGERING OF THE MANNOSE RECEPTOR ELICITS MATURATION OF MYELOID DENDRITIC CELLS SECRETING IL-10, NO IL-12 AND A DISTINCT CHEMOKINE PROFILE

M. Chieppa¹, G. Bianchi¹, A. Doni¹, A. Del Prete¹, M. Sironi¹, P. Monti², L. Piemonti², A. Biondi³, A. Mantovani^{1,4}, M. Introna¹ and P. Allavena¹

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Immature monocyte-derived dendritic cells (DC) strongly express the mannose receptor (MR). Addition of a specific anti-MR mAb (PAM-1) for 24h to cultures of immature DC induced phenotypical and functional maturation of these cells, assessed as upregulation of co-stimulatory molecules and CD83, low endocytosis of FITC-Albumin and chemotactic response to CCL19 (MIP3beta). A different isotype-matched anti-MR mAb, as well as anti-CD11c mAb, did not induce maturation. T cells co-cultured with PAM-1-matured DC initially proliferated, but later became unresponsive when rechallenged with fully mature CD40L-DC and behaved as suppressor cells in an independent MLR. DC stimulated with mAb PAM-1 expressed a distinct cytokine and chemokine profile: PAM-1-DC produced IL-10, especially after concomitant stimulation with LPS, low or undetectable IL-12 and were unable to polarize naive T cells into Th1 effectors. After stimulation with PAM-1, DC did not secrete CXCL10 (IP10) and CCL19 (MIP3beta) but produced large amounts of CCL22 (MDC) and CCL17 (TARC), thus favouring the amplification of Th2 circuits. Carbohydrate-rich proteins (mucins) derived from the gastrointestinal tract, tested as possible natural ligand of MR, bound to MR and induced IL-10 production in DC. In conclusion, appropriate triggering of the MR on DC activates a distinct maturation program characterized by the secretion of IL-10 and Th2-amplifying chemokines and low IL-12, thus providing a tolerogenic milieu for T lymphocytes. Selective engagement of the endocytic MR on myeloid DC may provide a new tool to tune adaptive immunity.

ROLE OF CTLA-4 IN TH SUBSET POLARIZATION

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²*Department of Biology, University of Rome "Tor Vergata", Rome, Italy*

T cell polarization of naïve CD4 cells to Th1 or Th2 cells is driven by IL-12 and IL-4, respectively. Nevertheless, other factors such as antigen type and concentration as well as costimulatory signals contribute to Th cell differentiation. CTLA-4 knock out mice show severe lymphoproliferative disorders, high levels of serum Ig and high frequency of IL-4- and IL-5-producing CD4 cells. We found that CTLA-4 engagement during Th cell polarization inhibits Th2 differentiation, as the frequency of IL-4-producing cells and IL-4 mRNA expression are reduced. In polarizing cultures CTLA-4 inhibits STAT6 activation and GATA3 expression, both factors being required for Th2 cell polarization. At variance, CTLA-4 does not affect Th1 polarization, as the frequency of IFN- γ -producing cells is comparable in experimental and control groups. Conversely, CTLA-4 engagement in polarized Th cells inhibits IFN- γ production in Th1 cells as well as IL-4 and IL-5 production in Th2 cells. Thus, CTLA-4 seems to play a different role by affecting polarization only to Th2 cell subset while controlling effector functions in both Th1 and Th2 cell subsets.

Session III
Adjuvants and cytokines
in infectious diseases

Chairpersons
Antonio Cassone - Barbara Ensoli

CYTOKINES AND TH1 VS TH2 RESPONSE IN HUMANS

Sergio Romagnani

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A large body of evidence has accumulated to suggest the existence of functionally polarized human T-cell responses based on their profile of cytokine secretion in the CD4⁺ T helper (Th)-cell subset. Type 1 Th (Th1) cells produce interferon (IFN)-gamma, interleukin (IL)-2 and tumor necrosis factor (TNF)-beta which activate macrophages and are involved in delayed-type hypersensitivity reactions. By contrast, type 2 Th (Th2) cells produce IL-4, IL-5, IL-10, and IL-13, which are responsible for strong antibody responses (including IgE) and inhibit several macrophage functions. Th1 responses preferentially develop during infections by intracellular bacteria, whereas Th2 cells predominate during infestations by gastrointestinal nematodes. Polarized Th1 and Th2 cells not only produce a different set of cytokines, which result in distinct functional properties, but also exhibit the preferential expression of some chemoattractant receptors. CCR4 and CCR8 are preferentially, and CRTH2 selectively, expressed by Th2 effector cells, whereas CXCR3 and CCR5 preferentially associate with Th1-like cells.

In addition to playing different roles in protection, polarized Th1-type and Th2-type responses can also be responsible for different types of immunopathological reactions in humans. Th1-dominated responses are involved in the pathogenesis of multiple sclerosis, type 1 diabetes mellitus, autoimmune thyroid diseases, Crohn's disease, *Helicobacter pylori*-induced peptic ulcer, acute allograft rejection, and some unexplained recurrent abortions. In contrast, Th2-type cells predominate in the "cumulus oophorus" and probably favor the occurrence of successful pregnancy by allowing the tolerance of fetal transplant, but they can be pathogenic in Omenn's syndrome, chronic graft-versus-host disease, idiopathic pulmonary fibrosis, progressive systemic sclerosis, and favor a more rapid evolution towards the full-blown disease in HIV-infected individuals. Moreover, allergen-specific Th2 responses are responsible for atopic disorders in genetically susceptible subjects.

A PUSH-PULL APPROACH TO MAXIMIZE VACCINE EFFICACY: AUGMENTING HELP AND ABROGATING SUPPRESSION WITH CYTOKINES, COSTIMULATORY MOLECULES, AND THEIR INHIBITORS

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We have attempted to optimize vaccine immune responses by a push-pull approach in which cytokines and costimulatory molecules are used to amplify and steer the response, and inhibitors of negative regulatory mechanisms are employed to relieve these dampening effects on the immune response. We used cytokines and costimulatory molecules emulsified with the antigen in an incomplete Freund's type adjuvant with a peptide HIV vaccine to provide a local depot for slow release to the same draining lymph nodes where the antigen is presented. GM-CSF and IL-12 have been found to amplify cytotoxic T lymphocyte (CTL) responses, and the two together are synergistic. Triple synergy was found with TNF-alpha added to GM-CSF and IL-12 that maximizes CTL responses and strongly steers to interferon-gamma-producing Th1 cells. Exploring the mechanism, we found that GM-CSF increases antigen presenting cell activity in the draining lymph nodes, and the combination of IL-12 and TNF-alpha markedly augments interferon-gamma production by increasing expression of the IL-12R β 2 receptor under low antigen conditions. A combination including the costimulatory molecule CD40L has also been found to be synergistic to optimize CTL responses. On the other hand, we have found that a negative regulatory mechanism involving IL-13 made by CD4⁺ NKT cells, dampens the CD8⁺ CTL response mediating tumor immunosurveillance and allows tumor recurrence. Abrogating this pathway with an inhibitor of IL-13 called IL-13R alpha2-Fc prevents tumor recurrence in mice. We asked whether this mechanism might dampen the CTL response to HIV peptide vaccines also, and whether we could increase the vaccine-induced CTL response by blocking this negative regulation. Indeed, administration of the IL-13 inhibitor or depletion of CD4⁺ cells increased the CTL response to an HIV peptide vaccine and improved the level of protection of mice against a challenge with a recombinant vaccinia virus expressing HIV gp160. We conclude that a push-pull approach in which CTL responses are amplified using a synergistic combination of cytokines and costimulatory molecules, and dampening mechanisms are abrogated with an inhibitor of IL-13, may allow optimal vaccine CTL responses and protection, especially for cancer and HIV in which the disease may contribute to suppressing the immune response.

INTERLEUKIN-2 ENHANCES HIV-SPECIFIC IMMUNE RESPONSES ONLY WHEN USED CONCOMITANTLY WITH THE ANTIGEN

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Background: IL-2, the first cytokine to be used in HIV therapy, is currently being studied in Phase II/III trials. IL-2 increases the CD4 count and decreases the latent HIV reservoir in resting CD4. It remains to be shown whether IL-2 could be used to reconstitute anti-HIV-specific immunity. We studied the immunomodulating activity of IL-2 and optimised the use of IL-2 to improve HIV-specific cellular immunity.

Methods: PBMC from 10 HIV-patients and 5 healthy donors, were stimulated for 15 hrs with Zinc-finger-inactivated HIV or mock antigen (lysozyme) and 50, 400, 1000 IU of rhIL-2. FACS analysis was performed after staining with antibodies anti CD8, CD4, CD3, CD45RA, CD45RO, IFN-gamma and perforin. $CD3/4/8VIR = \% \text{ of IFN-gamma producing } CD3/4/8^+ \text{ T cells after HIV stimulation} - \% \text{ of IFN-gamma producing } CD3/4/8^+ \text{ T cells after mock stimulation}$.

Results: IFN-gamma expressing cells were found in PBMC of HIV patients and not in healthy donor. CD3VIR was enhanced by IL-2 from 4.6% (50IU) to 10.4% (400IU) or to 10.9% (1000IU). No IFN-gamma producing cells were detected with mock antigen with any concentration of IL-2. The number of HIV-specific CD8⁺ T cell and CD4⁺ T cell doubled from 5.5% to 10.3% and from 3.7% to 6.7%, respectively. The amount of HIV-specific CD45RA⁺ T cell increased from 3.5% at 50IU to 6.3% at 400IU. The amount of HIV-specific CD45RO⁺ T cell increased from 8.7% at 50IU to 18.1% at 400IU. The percentage of perforin-containing HIV-specific CD8⁺ T cell increased from 3.5% to 8.3% and from 3.5% to 8.9% by increasing the IL-2 concentration from 50 to 400 and 1000 IU, respectively.

Conclusions: IL-2 is able to co-stimulate HIV-specific T cell responses. These data support the use of IL-2 in combination with vaccines, only when the antigen is given concomitantly with IL-2. The results have implication in the design of vaccines when this cytokine is used as adjuvant.

FLT3 LIGAND AND GM-CSF ADJUVANTS FOR HEPATITIS B VACCINATION OF HEALTHY ADULTS

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Three doses of hepatitis B vaccine are necessary to achieve high antibody titer in the general population, and thus many recipients fail to complete the required schedule. Both GM-CSF and recombinant human Flt3 ligand has been shown to improve responses to vaccination in animal models. Three trials were carried out using co-administered GM-CSF, and one trial using multiple doses of Flt3 ligand priming, in an effort to improve the response rate following the second vaccination. The GM-CSF was given as a single dose at the same site immediately following the hepatitis B vaccinations. The Flt3 ligand was given SQ on an every day or every other day dosing schedule. Despite easily measurable biologic effects, including increased white blood cell counts or increases in circulating dendritic cells, there was no measurable effect on antibody responses. Simple injection of these recombinant cytokines either at the site of vaccination (GM-CSF) or systemically (Flt3 ligand) is not efficacious in the human hepatitis B vaccination model.

PERTUSSIS TOXIN INDUCES MATURATION OF HUMAN MONOCYTE-DERIVED DC AND PROMOTES IL-12 DEPENDENT TH1 POLARIZATION OF NAIVE CORD BLOOD T CELLS IN SYNERGIC ASSOCIATION WITH LPS

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Objectives: pertussis toxin (PT) is a critically protective antigen present in all acellular pertussis vaccines, nevertheless the immunological mechanisms underlying protection from pertussis have not yet been elucidated. Several studies have emphasized the ability of PT to promote specific immune responses. Indeed it has been shown that PT induces T cell proliferation and that detoxified pertussis toxin (dPT) may act as a strong mucosal adjuvant. It has also been described that inoculation of PT in mice enhanced the capacity of splenocytes to produce IL-12 and that PT induces a healing phenotype in mice infected with *Leishmania major*, which is known to induce a Th1-dependent response.

Since dendritic cells (DC) exert a crucial role in the induction and regulation of the immune response, we investigated the capacity of PT and its non-toxic mutant to induce maturation and function of human monocyte-derived DC.

Results: Both native pertussis toxin (nPT) and dPT efficiently promoted human monocyte-derived DC surface expression of CD83, co-stimulatory molecules (CD80, CD86, HLA-DR) and alloreactive antigen presentation. nPT and dPT also showed able to induce production of several relevant cytokines, including IL-12 and IFN-gamma.

When DC were stimulated with PT plus LPS, while no differences were noticed in phenotypic maturation and overall cytokine production, IL-12 expression was markedly enhanced in a synergistic manner.

Soluble factors produced by LPS plus PT-stimulated DC induced Th1 polarization of naive cord blood T cells, fostering IFN-gamma but not IL-4 or IL-5 production.

Coherently, incubation of PT and PT plus LPS treated human monocyte-derived DC with anti-IL-12 mAb efficiently inhibited IL-12 and IFN-gamma production, alloreactive antigen presentation and naive cord blood Th1 polarization.

Conclusions: Our findings support the notion that nPT, besides its role in promoting specific immune response, is able to induce human monocyte-derived DC maturation and is a potent Th1 adjuvant; notably dPT has fully preserved this adjuvanticity. The synergism between PT and LPS in IL-12 production and Th1 polarization might be a relevant mechanism for vaccine-induced protection.

NEW ADJUVANTS

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Vaccines developed traditionally following empirical approaches have often limited problems of immunogenicity, probably due to the low level of purity of the active component(s) they contain. The application of new technologies to vaccine development is leading to the production of purer (e.g. recombinant) antigens which however tend to have a poorer immunogenicity as compared to vaccines of the previous generation. The search for new vaccine adjuvants involves issues related to their potential limits. Since the introduction of aluminium salts as vaccine adjuvants more than seventy years ago, only one adjuvant has been licensed for human use. This adjuvant, referred to as MF59, is contained in an influenza vaccine which has been licensed in Europe. The wide experience with this vaccine and the more recent experience with other vaccines given at a very young age, have shown that MF59 exhibits strong adjuvanticity and optimal safety profile. The development of some of these new vaccine adjuvants has been hampered by their unacceptable reactogenicity. In addition, some adjuvants work strongly with some antigens but not with others, thus limiting their potentially widespread use. The need to deliver vaccines *via* alternative routes of administration (e.g. the mucosal routes) in order to enhance their efficacy and compliance has set new requirements in basic and applied research to evaluate their efficacy and safety. CT and LT mutants given along with intranasal or oral vaccines are strong candidates as mucosal adjuvants. Their potential reactogenicity is still matter of discussions, although available data support the notion that the effects due to their binding to the cells and those due to the enzymatic activity can be kept separated. Finally, adjuvanticity is more often evaluated in terms of antigen-specific antibody titers induced after parenteral immunization. It is known that, in many instances, antigen-specific antibody titers do not correlate with protection. In addition, very little is known on parameters of cell-mediated immunity which could be considered as surrogates of protection. Tailoring of new adjuvants for the development of vaccines with improved immunogenicity/efficacy and reduced reactogenicity will represent one of the major challenges of the ongoing vaccine-oriented research.

TYPE I IFN AS A NATURAL ADJUVANT NECESSARY AND SUFFICIENT FOR A PROTECTIVE IMMUNE RESPONSE: LESSONS FROM THE INFLUENZA MODEL

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In this study, we found that several Th-1 promoting adjuvants (IFA, CFA or CpG oligonucleotides) induce type I IFN after injection in mice. We studied the kinetics of antibody production and the specific Ig isotype in both knock-out mice for type I IFN receptor (KO) and control animals after immunization with ovalbumin (Ova) in the presence or absence of adjuvants. KO mice showed a defective antibody response after immunization with the antigen mixed with adjuvants (IFA, CFA or CpG oligonucleotides), especially with regard to the production of IgG2a antibodies, and a defective Ova-specific T cell response, as revealed by *in vitro* 3H-thymidine uptake and *in vivo* DTH response. Then, we evaluated the capability of exogenous type I IFN to act as an adjuvant by using a commercially available influenza vaccine in normal mice. Co-injection of type I IFN with the vaccine resulted in a dose-dependent enhancement of the antibody response, especially with regard to Ig-G2a and IgA antibodies, which was associated with protection and long-term survival after inoculation of mice with influenza virus. Type I IFN proved to be equivalent to CFA in inducing protection from virus infection when used as an adjuvant of the influenza vaccine. After both intra-nasal and intra-muscular vaccination, type I IFN was superior with respect to MF-59 (a new adjuvant recently used for vaccination against influenza in elderly individuals) in inducing IgG2a and IgA antibodies. Since type I IFNs are the most used cytokines in medical practice, the findings that these factors are indispensable for the adjuvant-induced activity and sufficient for the generation of a protective immune response open new and practical perspectives in vaccine research and shed new light on the involvement of type I IFN in the early phases of the immune response.

TRANSGENIC PLASMODIUM KNOWLESI PRODUCES BIOACTIVE INTERFERON-GAMMA

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Recombinant microorganisms expressing host cytokines such as interferon gamma (IFN-gamma) have been shown to modulate immune responses leading to enhanced protection. However, modulation of malaria infection by cytokine-expressing parasites has not been examined. The recent development of transfection technology for malaria parasites facilitates expression of recombinant host immunomodulatory molecules such as cytokines. We explored the potential for manipulating host-parasite relationships in malaria using recombinant parasites expressing host cytokines. Therefore the expression of host IFN-gamma by *Plasmodium knowlesi*, a natural malaria parasite of macaque monkeys, was evaluated. The experimental host of *P. knowlesi*, *Macaca mulatta*, allows analysis of host responses to *P. knowlesi* in a system closely related to humans, immunologically well characterised and widely used as a model for vaccine development.

To engineer rhesus IFN-gamma-expressing *P. knowlesi*, various heterologous promoters from *P. berghei* were tested to control transgene expression in *P. knowlesi*, using firefly luciferase and green fluorescence protein as reporter molecules. The stage-specific *P. berghei* apical membrane antigen-1 promoter showed equivalent function in *P. knowlesi*. Using this promoter, we showed that *P. knowlesi* parasites transfected with expression plasmids for rhesus IFN-gamma produced bioactive IFN-gamma in a developmentally regulated manner. The expression did not directly influence growth of the parasites *in vitro*. Bioactivity of the parasite produced IFN-gamma was shown by inhibition of virus cytopathic effect and activation of *M. mulatta* peripheral blood mononuclear cells *in vitro*. Our data indicate for the first time that it is feasible to generate malaria parasites expressing bioactive host immunomodulatory cytokines. This offers opportunities to explore development of immuno-potentiated live parasite vaccines against malaria.

EFFICIENT DELIVERY OF T-CELL EPITOPES TO APC BY USE OF MHC CLASS II-SPECIFIC TROY-BODIES

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A major objective in vaccine development is the design of reagents that give strong, specific T-cell responses. We have constructed a series of recombinant Ab with specificity for MHC class II (I-E). Each has one of four different class II-restricted T-cell epitopes genetically introduced into the first constant domain of the heavy chain. These four epitopes are: 91-101 λ 2(315), which is presented by I-Ed, 110-120 hemagglutinin (HA)4 (I-Ed), 323-339 ovalbumin (OVA) (I-Ad), and 46-61 hen egg lysozyme (HEL) (I-Ak). We denote such APC-specific, epitope-containing Ab “Troy-bodies”. When mixed with APC, all four class II-specific Troy-bodies were ~1,000 times more efficient at inducing specific T-cell activation *in vitro* compared to non-targeting peptide-Ab. Furthermore, they were 1,000-10,000 times more efficient than synthetic peptide or native protein. Conventional intracellular processing of the Troy-bodies was required to load the epitopes onto MHC class II. Different types of professional APC, like purified B cells, dendritic cells (DC), and macrophages, were equally efficient at processing and presenting the Troy-bodies. *In vivo*, class II-specific Troy-bodies were at least 100 times more efficient at targeting APC and activating TCR transgenic T cells than were the non-targeting peptide-Ab. Furthermore, they were 100-100,000 times more efficient than synthetic peptide or native protein. The study shows that class II-specific Troy-bodies can deliver a variety of T-cell epitopes to professional APC for efficient presentation, *in vitro* as well as *in vivo*. Thus, Troy-bodies may be useful as tools in vaccine development.

Session IV
Cytokines and cancer vaccines:
what have we learned from animal models?

Chairpersons
Enzo Bonmassar - Guido Rasi

CYTOKINES AND DNA VACCINES IN THE PREVENTION OF HER-2/NEU CARCINOGENESIS IN BALB/C MICE

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The feasibility of preventing mammary cancer in individuals at risk by stimulating their immune system has been studied on female mice transgenic for the transforming rat Her-2/neu oncogene (BALB-neuT). All mammary glands of BALB-neuT mice progress to invasive carcinoma. Overexpression of rat p185 neu is first evident in the mammary gland of 3-week-old mice. Treatment of mice with preneoplastic lesions with systemic IL-12 delayed tumor onset and reduced tumor multiplicity. Carcinogenesis inhibition was associated with leukocyte infiltration, production of cytokines, reduction in microvessel number and necrosis. Leukocytes activated in the presence of IL-12 release factors able to arrest the cycle of endothelial cells, inhibit *in vitro* angiogenesis, and up-regulate ICAM-1 and VCAM-1 expression. Moreover, T-lymphocytes activated in the presence of IL12 modulate the genetic programs of tumor lines. cDNA gene expression array, RT-PCR, and protein expression showed that the expression of LPS-R, TTF1, TGF, and FGF, STAT-1, IRF-1, LMP-2, LMP-7, MIG, MCP-1, and Ang-2 genes was up-modulated, whereas that of VEGF, PA-28, IP-10, iNOS and MIP-2 genes was down-modulated. The opposite modulations of VEGF expression and of Ang-2, MIG, IP-10, and iNOS fit in well with the inhibition of angiogenesis that characterizes IL-12 antitumor activity. By changing its genetic programs, these factors also downmodulate the growth of tumor cells.

The ability of vaccination with plasmids coding for the extracellular and the transmembrane domains of rat p185neu to protect against Her-2/neu carcinogenesis was also evaluated. At 33 weeks of age control mice displayed palpable tumors in all mammary glands while about 60% of mice vaccinated on week 10 and 12 were tumor-free. While eventually these mice developed tumors at week 52, about 90% of BALB-neuT mice that received the two DNA vaccinations and were boosted with allogeneic cells expressing rat p185neu and releasing IFN-gamma were tumor free at week 52. At this time, mammary ducts were truncated, lined by a fibrotic stroma and an inflammatory infiltrate. Terminal buds were absent or heavily infiltrated by inflammatory cells. These findings suggest that the induction of a p185neu specific autoimmunity slowly lead to a progressive elimination of the preneoplastic cells responsible for tumor development.

CYTOKINES AND MOLECULARLY DEFINED ADJUVANTS CAN EITHER ENHANCE OR RESTRAIN THE EFFICACY OF RECOMBINANT CANCER VACCINES

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The identification and molecular characterization of tumor-associated antigens (TAA) recognized by T lymphocytes has made it possible to exploit genetic vaccination by inserting the TAA coding sequences into suitable bacterial or viral expression vectors. Preclinical animal models have shown that recombinant cancer vaccines expressing TAA fail to induce the strong immunity that was previously described against mouse tumors encoding model TAA. Indeed, many human and mouse TAA are viewed by the immune system as “self” antigens. A rational approach to amplifying the potency of gene-based vaccines is to use molecularly defined adjuvants such as cytokines. However, in selecting the best adjuvant it must be kept in mind that cytokines can trigger paradoxical reactions. High systemic levels of GM-CSF released by tumor cells or during intense immune stress can induce a temporary suppression of anamnestic T lymphocyte responses by recruiting myeloid suppressor cells in secondary lymphoid organs. IL-12 seems to discriminate negatively between “self” and “non self” since it abrogates the generation of functional CTL recognizing “self” TAA but not CTL directed against exogenous antigens. Moreover, molecular definition of the steps of T lymphocyte activation has generated classes of adjuvants that can affect different moments of the immune response, from the priming to the execution of the effector programs of each lymphocyte population and memory maintenance. Recent evidence suggests that molecularly defined adjuvants might be critically required for the preservation of autoreactive T lymphocytes directed against TAA, rather than the priming of antitumor lymphocytes. Finally, it is worth noting that potency enhancement of cancer vaccines might have an important cost: the autoimmune destruction of normal tissue.

IN VIVO TARGETING OF DENDRITIC CELLS USING CELLULAR VACCINES

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Our aim is to load dendritic cells directly *in vivo*. Two different strategies are tested:

1) DC bridges tumor cells- T cells interaction.

The use of tumor cells, as vaccine, is based on the rationale that they should retain the antigenic repertoire of a certain neoplasia. We have analyzed the effect of GM-CSF in combination with CD40 ligand (CD40L), since both molecules enhance DC functions. DC heavily infiltrated the C26 colon carcinoma transduced with both GM-CSF and CD40L. Tumor derived DC were able to stimulate a T cell clone specific for the C26 endogenous antigens. The result indicates that a professional APC can bridge the interaction between tumor cell vaccine and lymphocytes. Another combination tested the effect of GM-CSF on DC and of OX40L on T cells. C26 cells transduced with both GM-CSF and OX40L were rejected by 90% of the injected mice. FACS analysis of tumor infiltrating leucocytes showed a reduction in number of GR1⁺ cells and an increase of CD11c⁺ DC.

2) Secretion of heat shock proteins (hsp) by engineered tumor cells.

Hsp are associated with antigenic peptides of the cellular antigens and, when released by dead cells, they act as a danger signal inducing DC maturation. To release hsp in the extracellular milieu, independently from cellular death, we produced a vector, Signal-phsp70-Ck, that induces the secretion of the corresponding hsp fused with the constant domain of the murine Ig K chain. Different murine tumor lines were transfected with this vector and their immunogenicity was tested in syngeneic hosts. Immunization with hsp70Ck secreting tumors was more potent in inducing a cytotoxic response *in vitro* than immunization with the parental tumor cells. The immune response was specific and directed to an immunodominant epitope associated to the murine carcinomas employed in the assay, thus suggesting that hsp70Ck, redirected from cytoplasm to the secretory pathway is an effective carrier for peptide precursors of corresponding tumor antigens.

DENDRITIC CELLS FROM BENCH TO BEDSIDE

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Dendritic cells have the unique capacity to attract, interact and activate naive T cells to initiate immune responses. Understanding the molecular mechanisms underlying the efficient induction of immunity by DC is essential to optimally use DC in f.e. DC-based vaccines. Using different approaches we have identified a number of antigens preferentially expressed by DC. Two of them have been investigated in further detail: a chemokine termed DC-CK-1, that preferentially attracts naive T cells and B cells, and DC-STAMP, a novel multimembrane spanning receptor. Next to their role in DC-biology, mAbs directed against these novel antigens are useful markers for DC/DC-subsets *in vivo*. The capacity of DC to initiate immune responses allows their exploitation in therapeutic strategies against cancer and other diseases. DC-migration studies and the efficacy of DC-based vaccines in comparison to alternative vaccination strategies are investigated in mouse models. Using the fully autologous mouse TRP-2/B16 tumor model, we demonstrated that vaccination with TRP-2 peptide-loaded bone marrow-derived dendritic cells results in activation of high avidity TRP-2-specific CTLs and protective immunity against a lethal challenge with wildtype B16. Interestingly, in spite of the observed CTL-mediated melanocyte destruction *in vitro*, melanocyte destruction *in vivo* was sporadic and primarily restricted to minor depigmentation at the vaccination site. Strikingly, within this autologous model, vaccination with the same TRP-2 peptide in Freund's adjuvant or TRP-2-encoding plasmid DNA did not result in protective immunity against wildtype B16. These results emphasize the potency of DC-based vaccines to induce immunity against autologous tumor-associated antigens. We initiated clinical studies in which we evaluate 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 first 20 patients entered in the clinical trials, and the immuno-monitoring data indicate that mature DC are far superior to immature DC to induce immune responses *in vivo*.

ENHANCING ANTI-TUMOR IMMUNITY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION

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The anti-tumor role of the immune system has been well documented in patients undergoing an HLA-identical allogeneic bone marrow transplant. However, what is gained in anti-tumor response is lost by the significant toxicity of the graft vs host disease resulting in persistent non-specific immune stimulation. In contrast, autologous transplants have traditionally been viewed simply as a platform to deliver high-dose chemotherapy void of any immune-mediated therapeutic benefit.

However, despite the significant immunosuppression characterizing the immediate post-transplant period, several theoretical advantages exist that make the autologous transplant setting an ideal platform upon which to integrate immunotherapeutic strategies. 1. It is a setting of minimal tumor burden in which active vaccination has been demonstrated to have its greatest efficacy. 2. The myeloablative regimen can abolish intrinsic tolerogenic mechanisms thereby restoring vaccine responsiveness. 3. The infusion of large numbers of T cells at the time of stem cell infusion facilitates the adoptive transfer of immunity. 4. Vaccination during the period of immune reconstitution can result in skewing of the developing T cell repertoire towards greater recognition of tumor antigens of interest.

Using the A20 lymphoma modified to express the model tumor antigen, influenza hemagglutinin (A20HA) and I-Ed restricted HA-specific CD4 T cells, we have been able to track the fate of tumor-specific T cells during the course of tumor progression. Significant activation and Th1 skewing of HA-specific T cells was observed three weeks after transplantation of A20HA-bearing mice. In sharp contrast, analysis of the clonotypic HA-specific T cells at the same time point following adoptive transfer into non-transplanted tumor-bearing recipients showed profound functional impairment. Furthermore, the adoptive transfer of fully tolerant T cells from a tumor-bearing donor to a tumor-bearing transplanted host resulted in marked T cell activation as measured by clonotypic expansion and γ -IFN production. The ability to revert from a tolerant to an active state may be a critical property of autologous transplantation possibly driven by multiple factors. Taken together, this data demonstrates several unique immuno-modulatory features of the autologous transplant setting. Studies examining the underlying mechanisms are underway.

INTERFERON-GAMMA-INDEPENDENT RESPONSES INDUCED BY MAMMARY ADENOCARCINOMA CELLS ENGINEERED WITH IL-15 AND IL-12 GENE IN SYNGENEIC MICE

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IL-15 and IL-12 display anti-tumor activity in different tumor models and synergistic effects have been described both *in vitro* and *in vivo*. IFN-gamma appeared to be a secondary mediator of IL-12 and IL-15 effects and also of their cooperative action *in vivo*. TS/A murine adenocarcinoma cells were engineered to secrete IL-12, IL-15 or both cytokines by the use of plasmid expression vectors. TS/A cells secreting IL-15 (TS/A-IL-15) displayed a reduction of tumorigenicity (50%) when implanted s.c. in syngeneic mice, while both TS/A IL-12 and TS/A-IL-12/IL-15 were rejected by 100% of syngeneic animals. Differently, TS/A-IL-15 and TS/A-IL-12 were tumorigenic in syngeneic IFN-gamma knock out (GKO) mice (100 and 90% of take, respectively), while TS/A-IL-12/IL-15 were completely rejected by 90% of GKO mice. GKO mice rejecting TS/A-IL-12/IL-15 developed protective immunity against TS/A antigens, as indicated by rejection of wild type TS/A cells upon re-challenge. Immunohistochemical analysis of the tumor area in GKO mice during the rejection of TS/A-IL-12/IL-15 showed an increased number of CD8⁺ T cells, NK cells, granulocytes, macrophages and dendritic cells and a sustained production of GM-CSF, TNF-alpha, MCP-1 and MIP-2 as secondary cytokines. GKO splenocytes co-cultured with TS/A-IL-12/IL-15 showed an increased secretion of GM-CSF and TNF-alpha. *In vivo* depletion experiments showed that the response to TS/A-IL-12/IL-15 cells required CD8⁺ T lymphocytes, while NK cells, granulocytes and CD4⁺ cells played a minor role. In addition, an anti-TNF blocking mAb delayed and partially inhibited the rejection process. These data show synergistic effects of IL-12 and IL-15 in anti-tumor responses in IFN-gamma KO mice that are related to the induction of other cytokines, such as TNF, and chemokines. CD8⁺ cells play an essential role in this response. A microarray approach is under investigation to further identify additional IFN-gamma independent mediators of the IL12+IL-15 action.

Session V
Clinical use of cytokines in vaccine development:
state of art and perspectives

Chairpersons
Giuliano D'Agnolo - Robin Foà

CYTOKINES IN CANCER VACCINES

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Studies using animal models have demonstrated that transduction of genes encoding different cytokines into tumor cells results in a local recruitment of inflammatory cells that in turn can inhibit tumor growth. This is often accompanied by tumor antigen priming of the host immune system, which becomes resistant to subsequent challenge by the parental, untransduced tumor. Gene-transduced tumor cells have therefore been widely used as vaccines, although in the therapeutic setting their antitumor efficacy was limited to a few animal models. On the basis of this rationale, clinical studies were initiated, results of which will be summarized to identify the reason for their limited efficacy. I will present results of our studies in melanoma with cytokine and B7-1 gene-modified melanoma cells. Moreover, I will point out problems generated by the use of autologous versus allogeneic gene-transduced vaccines, by the choice of the appropriate cytokine(s), and by patient selection. Results of these studies will also be compared with those obtained by peptide-based vaccines in similar groups of patients. Altogether, we conclude that improvements can be made in the construction of gene-modified vaccines by (1) using tumor cells known to express molecularly defined antigens, (2) introducing, in addition to gene encoding cytokines, genes encoding T cell costimulatory molecules, (3) increasing the amount of cytokine released locally by irradiated cells, and (4) coadministering adjuvant cytokines (IL-2 and IL-12) systemically in order to expand the T cell pool activated by vaccines.

HIGH-DOSE INTERFERON (HDI) – CORNERSTONE OF PRESENT ADJUVANT THERAPY FOR MELANOMA, AND A PLATFORM FOR FUTURE PROGRESS

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HDI is the first adjuvant regimen demonstrated to prolong survival in high-risk melanoma and licensed worldwide on the basis of ECOG's trial E1684 (Kirkwood, *et al. J Clin Oncol* 1996;14:7). This experience was amplified in the published E1690 and E1694 intergroup trials (Kirkwood, *et al. J Clin Oncol* 2000 ;18:2444; Kirkwood, *et al. J Clin Oncol* 2001;19:2370). E1694 constitutes the largest randomized trial of adjuvant systemic HDI therapy ever conducted, and demonstrated reduction of relapse rate for HDI over the GM2-KLH-QS21 vaccine that is consistent with the prior E1684 and E1690 trial results, in which HDI was compared to observation. A durable 33% reduction in the likelihood of relapse and death led to the early closure of E1694 at 1.6 years of median follow-up by the external data safety committee. The results of E1694 updated to 2.1 years median follow-up are unchanged. Analysis of the pooled patient data for 1912 patients who entered trials of HDI conducted in cooperative groups (Kirkwood, *et al. J Clin Oncol* 2001;19:1430) confirmed the benefit of IFN. Toxicity requires discontinuation of therapy in 25% of patients but the management of IFN-associated toxicity has improved (Kirkwood JM, *et al. unpublished*). Efforts to reduce the toxicity of HDI, and to enable combination with other agents focused upon two strategies: 1) the evaluation of induction IV HDI for 1 month alone (E1697), in intermediate-risk resected melanoma patients; and 2) evaluation of PEG-conjugated IFN that may improve the kinetics of delivery for long-term therapy (E3601). Modulation of immune responses to vaccines by HDI was evaluated in relation to the antibody-inducing vaccine GM2-KLH-QS21, in E2696 (Kirkwood, *et al. J Clin Oncol* 2001;19:1430), and in terms of multi-peptide vaccines that induce T cell response to lineage antigens of melanoma, in E1696. E2696 showed no inhibition of antibody responses by HDI, permitting consideration of HDI and antibody-inducing vaccines (Kirkwood, *et al. J Clin Oncol* 2001;19:1430). Current studies of IFN, GM-CSF and other dendritic cell-promoting cytokines with peptide vaccines are a major focus of current investigations. The influence of IFN α 2b, GM-CSF, and these cytokines with a multi-epitope peptide vaccine (Melan-A/MART-1 27-35, gp100 209-217(210M), and tyrosinase 368-378(370D)) is the goal of E1696. The same 3 lineage antigen peptides studied in E1696 are under adjuvant exploration with GM-CSF for patients with resected advanced stage III or isolated stage IV disease in E4697. Bio-chemotherapy with IFN and IL-2 is being compared to HDI for adjuvant therapy of high-risk stage IIIB-C patients in the Intergroup trial S0008. Pre-operative application of HDI is also under study (UPCI 00-008). The analysis of HDI promises to reduce its toxicity, and to improve its efficacy and therapeutic index.

PERSPECTIVES IN THE USE OF TYPE I IFN AND DENDRITIC CELLS FOR THE DEVELOPMENT OF HUMAN VACCINES

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Type I IFNs (especially IFN- α) are the most used cytokines in patients with malignancies and viral diseases. In spite of many years of intense studies, only recently we have begun to understand how effects of these cytokines on specialized cells of the immune system may play an important role in their antitumor and antiviral activity. The recent discovery of strong correlations between efficacy in patients and induction of selective immune responses is consistent with concepts stemming from early studies in mouse tumor models using type I IFNs or genetically modified cells producing IFN- α . An ensemble of recent studies in mouse and human models have revealed novel effects of type I IFNs on T cells and dendritic cells, which underscore the importance of these cytokines in linking innate and adaptive immunity against infections and neoplastic diseases. Notably, type I IFNs have been recently shown to act as a powerful vaccine adjuvants when coinjected with definite immunogens in mice. In addition, recent results indicate that these cytokines can promote the rapid differentiation of human monocytes into highly active DCs (IFN-DCs), capable of inducing a Th-1 type of humoral and cellular immune response and antigen-specific CD8⁺ cells *in vitro* as well as in hu-SCID-mouse models. Altogether, these results strongly support the concept of using type I IFNs as adjuvants for the development of new or more effective human vaccines. The perspectives for the use of type I IFNs as vaccine adjuvants as well as the possible advantages of using IFN-DCs in strategies of immunotherapy of cancer and HIV-1 infection will be presented and discussed.

CANCER VACCINATION WITH PEPTIDES AND GRANULOCYTE MACROPHAGE STIMULATING FACTOR (GM-CSF)

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Tumor-associated antigens recognized by cellular or humoral effectors of the immune system represent potential targets for antigen-specific cancer immunotherapy. Different categories of cancer antigens have been identified which induce CD8⁺ cytotoxic T lymphocyte responses *in vitro* and *in vivo*: 1) 'Cancer Testis' (CT) antigens, expressed in different tumors and normal testis, 2) melanocyte differentiation antigens, 3) point mutations of normal genes, 4) 'self'-antigens that are overexpressed in malignant tissues, and 5) viral antigens. Clinical studies with peptides and proteins derived from these antigens have been initiated to study the efficacy of inducing specific CD8⁺ T cell responses *in vivo*. Immunological and clinical parameters for the assessment of antigen-specific immune responses have been defined, i.e. DTH-, CD8⁺ T cell-, autoimmune-, and tumor regression responses. Specific DTH- and CD8⁺ T cell responses and tumor regressions have been observed after intradermal administration of tumor-associated peptides alone. Peptide-specific immune reactions were enhanced after using GM-CSF as a systemic adjuvant by increasing the frequency of dermal antigen-presenting Langerhans cells. Complete tumor regressions have been observed in the context of measurable peptide-specific CD8⁺ T cells. However, in single cases with disease progression after an initial tumor response, either a loss of single antigens targeted by CD8⁺ T cells or of the presenting MHC class I allele was detected, pointing towards immunization-induced immune escape. Ways to modulate antigen- and MHC class I expression *in vivo* are being evaluated to prevent immunoselection. Recently, a new CT antigen, NY-ESO-1, has been identified on the basis of spontaneous antibody responses to tumor-associated antigens (SEREX). NY-ESO-1 appears to be one of the most immunogenic antigens known today with spontaneous immune responses observed in 50% of patients with NY-ESO-1 expressing cancers. Clinical studies have been initiated to evaluate the immune responses to vaccination with different NY-ESO-1 peptides combined with GM-CSF in relation to the clinical development.

CROSS-PRIMING OF TUMOR-SPECIFIC T CELLS IN VITRO AND IN MELANOMA PATIENTS USING AUTOLOGOUS DENDRITIC CELLS AS NATURAL ADJUVANT

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Dendritic cells were produced from unfractionated leukapheresis products using IL-13 and GM-CSF (Dendritophages). Freeze/thaw lysates from allogeneic melanoma cell lines were used as a source of antigens to load these Dendritophages. Loaded and matured Dendritophages were shown to stimulate *in vitro* the differentiation of purified CD8⁺ T cells into cytotoxic effectors secreting interferon (IFN)-gamma in response to tumor-shared antigens such as Melan-A/MART-1 and gp100.

A pilot clinical trial was initiated in stage IV melanoma patients. Patients received subcutaneous, intradermal and intranodal injections of autologous Dendritophages loaded with an allogeneic melanoma lysate. Vaccinations were remarkably well tolerated. Using ELISPOT, tetramers and Immunoscope, immune responses specific for Melan-A/MART-1 and gp100 were detected in the blood of vaccinated patients. Noteworthy, signs of clinical response were observed in several patients after vaccination.

IMMUNOGENIC ROLE OF THE APOPTOSIS INHIBITOR PROTEIN SURVIVIN IN COLO-RECTAL CANCER PATIENTS

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Survivin is a recently characterized mammalian IAP (Inhibitor of Apoptosis Protein), over-expressed in most human cancers but practically undetectable in normal differentiated tissues; due to its cell-cycle-regulated and tumor-specific expression, it is believed to play a crucial role in oncogenesis and could be considered as a shared and tumor-specific antigen.

It has been recently shown that survivin-specific CD8⁺ T cells could be generated *in vitro* by stimulating PBMCs from melanoma and leukaemia patients with survivin-derived peptides and that, in some cases, a spontaneous cytotoxic T-cell response could be evaluated.

Therefore, survivin could be considered as an optimal candidate for anticancer immunotherapeutic strategies. To this aim, we evaluated its capacity to elicit a specific T-cell reactivity against survivin-derived epitopes in peripheral blood and tumour-associated lymphocytes (TALs) from colorectal cancer patients.

We identified an HLA-A2 restricted, survivin-derived epitope that was first evaluated for its ability to restore the HLA-A2 expression in the TAP-deficient T2 cell line. T cells from colorectal cancer patients were subjected to two or three cycles of stimulation by autologous PBMCs loaded with the identified peptide, named SVV-1. We could detect a survivin-specific reactivity in three of ten colorectal cancer patients. From two of the ten patients, TALs obtained at the time of the surgery were stimulated with the same peptide and analyzed after 4 week of stimulation with the same peptide.

In all cases, stimulated T cells were also able to recognize a naturally processed, SVV-derived epitope expressed by B-lymphoblastoid, SVV-transfected C1R-A2 cell line (C1R-A2/SVV).

Finally, we could measured a HLA class II-restricted, survivin-specific response in PBLs from cancer patients subjected to several cycles of stimulation by autologous PBMCs pulsed with soluble recombinant survivin protein.

Our data therefore indicate a possible immunological role of survivin in colorectal cancer patients and allow us to consider survivin as a potential candidate for cancer immunotherapy.

EX VIVO PRIMING AND EXPANSION OF DONOR-DERIVED LONG-TERM ANTI-LEUKEMIA CTL LINES

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We previously demonstrated that it is possible to induce long term AML-specific MHC class I-restricted CD8 T cells, using CD8-enriched lymphocytes and dendritic cells (DC) obtained from HLA-matched allogeneic bone marrow transplantation donors and apoptotic leukemia blasts obtained from the BM recipients as the source of tumor antigen. AML-specific CTL lines displayed high cytotoxic activity towards recipient leukemic blasts (LB), but very low or absent (< 10%) reactivity against patient non-leukemic cells (Montagna, *et al. Blood* 2001;98:3359). In the present study, we evaluated the possibility i) to generate anti-leukemia specific CTLs directed against tumor cells other than AML blasts and ii) to use lymphocytes and DC derived from HLA-mismatched donors as effector cells for the induction of these lines. At present, seven donor/recipient pairs were analyzed. Two of 7 patients were affected by ALL, 1/7 by JMML, and 4 by AML. Five of 7 patients received allogeneic BMT from HLA-mismatched donors. Anti-leukemia CTL lines were obtained from 6 out of the 7 donor evaluated. The donor who failed to induce specific CTL lines showed a marked reduction in the capacity of adherent mononuclear cells to differentiate *in vitro* towards monocyte-derived DC. CTL lines obtained in this set of experiments showed LB-directed cytotoxic and expansion capacity similar to those previously described. Levels of alloreactivity, detected only in 2 out of 5 mismatched donors, were sizeable but lower than anti-leukemia activity at all effector/target ratio tested. These experiments demonstrated that the methodological strategy previously employed to generate anti-AML CTL lines is also suitable in case of leukemia cells other than AML blasts and can be applied also in the contest of HLA-mismatched donor/recipient pairs. Further experiments are in progress to evaluate the frequency of cytotoxic precursor cells able to maintain long-term expansion of antileukemia T cell lines.

Poster abstracts

1 ACTIVATION OF SRC FAMILY TYROSINE KINASES BY LPS IS REQUIRED FOR FULL DENDRITIC CELL MATURATION

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Activation of DCs by specific stimuli, such as pathogen components or inflammatory mediators, to undergo a specific process of maturation is a fundamental step in the initiation of an effective immune response. Among the most potent initiators of DC maturation is the cell-wall component of Gram-negative bacteria, the lipopolysaccharide (LPS). Recently the signaling events triggered by LPS have been partially elucidated and shown to involve the activation of Toll like receptors (TLRs), ultimately resulting in NF- κ B transactivation. Besides, LPS has been shown to trigger other signaling events such as the activation of the MAP kinases ERK, p38 and JNK. Using specific inhibitors of the different biochemical cascades we have addressed the role played by each of the signaling pathways triggered by LPS on the maturation process in human DCs, by analyzing several parameters associated with DCs activation. LPS induced the activation of the Src family kinases c-src and Lyn. Block of these enzymes by treatment with the specific tyrosine kinase inhibitor PP1 was not accompanied by changes in the expression levels of typical DC maturation markers such as CD80, CD83, CD86, HLA-DR. However, production of the inflammatory cytokines IL-6, IL-12, MCP-1 and TNF- α was prevented. Consistent with this result LPS-induced expression of IRF-1, a transcriptional regulator of these cytokines, was prevented by PP1 treatment. Interestingly PP1 also inhibited LPS-induced chemokine receptors switch as demonstrated by the high and low expression levels of CCR5 and CCR7, respectively, in PP1 treated DCs. These effects were specific to the Src family kinases since PP1 did not affect the other biochemical cascades triggered by LPS, in particular the JNK, NF- κ B, p38, ERK pathways. We then addressed whether the dissociation of the induction of the inflammatory cytokines from the other maturation changes affects the capacity of the DCs to induce a Th1/Th2 differentiation in naïve T cells. Indeed inhibition of Src family kinases during activation resulted in DCs that are less efficient in inducing Th1 differentiation. This finding suggests that it is possible to modulate the class of immune response induced by DCs.

2 INTERACTIONS BETWEEN NATURAL KILLER AND DENDRITIC CELLS: A MODEL FOR A FEEDBACK CONTROL OF THE IMMUNE RESPONSE

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During the innate response to many inflammatory and infectious stimuli, dendritic cells (DCs) undergo a differentiation process termed maturation. Mature DCs activate antigen-specific naïve T cells.

Here we show that both immature and mature DCs activate resting human Natural Killer (NK) cells. Within one week the NK cells increase 2-4 fold in numbers, start secreting IFN γ and acquire cytolytic activity against the classical NK targets. The DC-activated NK cells then kill immature DCs efficiently, even though the latter express substantial levels of MHC class I. Similar results are seen with IL-2-activated NK cell lines and clones, i.e., these NK cells kill and secrete IFN γ in response to immature DCs. Mature DCs are protected from activated NK lysis, but lysis takes place if the NK inhibitory signal is blocked by an HLA-A,B,C specific antibody.

The NK activating signal received by DCs derived from monocytes mainly involves the NKp30 Natural Cytotoxicity Receptor, but neither the NKp46 or NKp44 receptor nor the 2B4 coreceptor. Conversely, a subset of DCs derived from CD34⁺ hemopoietic cell precursor was recognized through NKp30, NKp44 and NKp46. The activating signal mediated by these receptors was enhanced by the engagement of 2B4. This was consistent with the expression of CD48, the major ligand of 2B4, on the subset of CD34⁺ cell-derived DCs but not on monocyte-derived DCs. However, DCs seem to use a NKp30, NKp44 and NKp46 independent mechanism to stimulate proliferation of resting NK cells.

We suggest that DCs are able to control directly the expansion and the activation of NK cells and that the lysis of immature DCs can regulate the afferent limb of innate and adaptive immunity. These results are also consistent with the hypothesis that CD48⁺ DCs may represent a distinct subset of DCs with different functions and/or ontogeny.

3. INTERFERON- γ AND PERFORIN SECRETION ELICITED IN NK CELLS BY CONTACT WITH DENDRITIC CELLS IS TRIGGERED BY DIFFERENT PATHWAYS DEPENDING ON THE MOLECULE ENGAGED DURING ADHESION

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We have described that the engagement of the natural cytotoxicity receptors (NCR) NKp30 and NKp46 by their natural ligands expressed on dendritic cells (DC), triggers the cytolytic machinery of autologous natural killer (NK) cells (*Eur J Immunol* 2001;31:1656). NK cell-mediated lysis of autologous DC is dependent on the activation of the phosphatidylinositol-3-kinase (PI-3K) and its substrate Akt/PKB. Interestingly, binding to autologous DC induces a calcium influx in NK cells, followed by activation of the calcium-calmodulin kinase II (CAMKII), release of perforin and granzymes and interferon- γ (IFN- γ) secretion. CAMKII is induced *via* LFA-1: indeed, oligomerization of LFA-1 leads to CAMKII induction in NK cells. Moreover, release of lytic enzymes and cytotoxic activity are strongly reduced by masking LFA-1 or by adding CAMKII inhibitors such as KN62 and KN93, at variance with the inactive compound KN92.

NK cell mediated lysis of DC, and IFN- γ release by NK cells upon NK/DC contact, are inhibited by exogenous HIV-1 Tat: the protein blocks calcium influx and impairs CAMKII activation elicited *via* LFA-1 in NK cells, eventually inhibiting degranulation. Experiments performed with synthetic, overlapping Tat-derived peptides showed that the C-terminal domain of the protein is responsible for inhibition. Finally, both KN62 and Tat reduced the extension of NK/DC contacts, possibly affecting NK cell granule polarization towards the target. These data provide evidence that exogenous Tat inhibits NK cell activation occurring upon contact with DC: this mechanism might contribute to the impairment of natural immunity in HIV-1 infection.

4 RHO FAMILY PROTEINS AS EARLY REGULATORS OF DENDRITIC CELL POLARITY AFTER CYTOKINE-INDUCED DIFFERENTIATION

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Dendritic cells (DCs) are antigen presenting cells able to generate a primary T cell response. They are considered as essential regulators of both innate and acquired immune response. In addition to their phenotypic and functional features, once differentiated in mature cells, they show a specific cell morphology characterized by thin and long dendritic-like protrusions. DCs need thus a dynamic organization of cytoskeleton capable of a flexible activity leading to their typical morphology. Furthermore, cell locomotion and motility represent functional characteristics of importance in exerting a proper in situ activity by these cells. To do this, a series of structural alterations are exploited by DCs during differentiation leading to changes in cell adhesion and spreading pattern. Consequently, cell-cell (homotypic) and cell-substrate (heterotypic) interactions are deeply modified. Cell adhesion and cell spreading are well defined and different phenomena which are related to several intracellular structures or molecules including the actin cytoskeleton. The actin microfilament integrity and function are in fact prerequisites for both the events. It is now clear that F-actin cytoskeleton organization is controlled by proteins belonging to the Ras-like p21 Rho family. The Rho family of small GTPases encompasses three different subfamilies, all controlling the actin cytoskeleton: Rho subfamily induces stress fibers assembly, Rac the ruffling activity, and Cdc42 the filopodia extension. Interestingly, some evidence has previously indicated that these molecules can play a role in the morphogenic changes leading to DC activity and function. In the present work, we analyzed human DCs generated from monocytes in the presence of GM-CSF and two different triggers, i.e. IL-4 and IFN- α , with the aim of pointing out the behavior of Rho family proteins, rho, rac and cdc 42, during the DC differentiation. We found: i) early qualitative and quantitative changes of rho and rac molecules but not of cdc42; ii) a sequential rearrangement of these molecules during differentiation, iii) an increased expression of rho-mediated cell-cell adhesion molecule E-cadherin. Altogether these findings seem to suggest a role for IL-4 and IFN- α in the modulation of rho family proteins which, in turn, may act as supervisors of DC polarity, locomotion and spreading.

5 INTERFERON- α AS THE NATURAL ADJUVANT FOR THE PROMPT GENERATION OF HIGHLY ACTIVE DENDRITIC CELLS FROM PRE-ARMED CD2⁺ MONOCYTES

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Culture of freshly isolated human monocytes in the presence of type I IFN and GM-CSF resulted in the rapid generation of highly active dendritic cells (DCs) (*J Exp Med* 2000;191:1777; *Blood* 2001;98:3022). These DCs (IFN-DCs) were endowed with potent functional activities, not only *in vitro* but also *in vivo*, as evaluated by their strong capability to induce the generation of a primary human antibody response and antigen specific CD8⁺ T cells in hu-PBL-SCID mice. Notably, IFN-DCs promoted a Th-1 type of immune response and showed the phenotype of partially mature DCs, as revealed by the detection of specific markers (CD83, CD25, CCR7), expression of cytokines (IL-15) and chemokines (IP-10) and migratory potential. More recently, we have studied the role of IFN in the early phases of differentiation of monocytes into DCs. Since DCs represent the main players linking innate and adaptive immunity, their prompt generation from blood cells would be instrumental for an efficient immune response to infections. Consistent with this, CD2⁺ monocytes were found to express the DC maturation marker CD83, along with acquisition of high antigen presenting activity, after a surprisingly short time (i.e., 4 hours) in culture. Exposure of monocytes to IFN- α , but not to IL-4, induced persistence of CD2⁺/CD83⁺ cells, which were fully competent in stimulating primary response by naive T cells. These results unravel the natural pathway by which infection-induced signals rapidly transform pre-armed monocytes into active DCs.

6 LOSS OF TYPE I INTERFERON RECEPTORS AND IMPAIRED INTERFERON RESPONSIVENESS DURING TERMINAL MATURATION OF MONOCYTE-DERIVED HUMAN DENDRITIC CELLS

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Type I interferons (IFNs) are modulators of myeloid dendritic cell (DC) development, survival and functional activities. Here, we monitored the signal transduction pathway underlying type I IFN biological activities during *in vitro* maturation of human monocyte-derived DCs. IFN-inducible tyrosine phosphorylation of signal transducer and activator of transcription (STAT) family members was found to be severely impaired upon lipopolysaccharide (LPS)-induced DC maturation. This correlated with a marked down-modulation of both type I IFN receptor chains occurring as early as 4 h after LPS treatment. The reduced receptor expression was a post-transcriptional event only partially mediated by ligand-induced internalization/degradation. In fact, although an early and transient production of type I IFNs was observed after LPS treatment, its neutralization was not sufficient to completely revert the IFN receptor down-modulation. Notably, the neutralization of the LPS-induced, endogenous type I IFNs did not interfere with the acquisition of a fully mature surface phenotype, nor had a significant effect on the allostimulatory properties of LPS-stimulated DCs. Overall, these data indicate that DCs strictly modulate their responsiveness to type I IFNs as a part of their maturation program, underlining the importance of the IFN system in the regulation of DC physiology.

7 TUMOR CELL LYSATE-LOADED DENDRITIC CELLS INDUCE A T-CELL RESPONSE AGAINST COLON CARCINOMA CELLS

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Identification of an efficient Ag loading strategy remains one of the critical challenges in dendritic cell-based vaccination protocols. Contrary to the peptide-based approach, the use of crude tumor material can target multiple specificities, thus reducing the risk of tumor escape variants. Immune strategies explored so far in the cure of colon cancer have shown a superior, although modest, host response after vaccination with whole tumor cells compared to CEA-loaded DC. Native tumor antigens can be delivered to DC as cell lysates or apoptotic cells. In a previous study we showed either stimulatory or suppressive responses by colon cancer patients' lymphocytes stimulated *in vitro* by DC loaded with the autologous apoptotic tumor. In the present study we have addressed the response of colon cancer patients to tumor lysate-pulsed DC. DC, derived from GM-CSF and IL-4 cultured blood monocytes, were exposed to lysates from autologous tumor (T-DC) or peritumoral normal mucosa (NM-DC), stimulated for 24 h with TNF- α to achieve full DC maturation (assessed by MHC II and CD86 expression) and cultured with autologous lymphocytes in the presence of IL-7 and IL-2. Development of lymphocyte tumor-specific response was evaluated in IFN- γ ELISPOT assays during a 12 hr incubation with the autologous tumor, normal mucosa or T-DC, in the presence/absence of an anti-class I MHC mAb. Specific sensitization against the relevant tumor antigen was observed in 3 out of 5 patients. In these patients the antitumor response was higher than that induced by the PKC activator PMA plus ionomycin, which was below normal levels. Conversely, very high response to PMA-ionomycin, low response to tumor and inhibition by the sensitizing normal cells were observed in cultures stimulated with NM-DC. Increased proliferation was observed in T-DC, compared to NM-DC stimulated cultures. Optimal proliferation and tumor antigen commitment of CTL progenitors required three rounds of stimulation or magnetic beads selection of IFN- γ -producing cells followed by their expansion on a feeder layer. The observation that colon carcinoma cells can induce a tumor specific CD8 response when delivered as cell lysates to *in vitro* generated DC may be relevant to the design of therapeutic vaccines.

8. A RECOMBINANT BACTERIOPHAGE VECTOR FOR ANTIGEN-PRESENTING CELL (APC)-BASED IMMUNOTHERAPY OF HUMAN CARCINOMAS

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We have previously showed that bacteriophage fd, displaying peptide epitopes from HIV-1, elicited strong cytolytic responses. Here, we have investigated the ability of APCs pulsed with double displaying bacteriophage fd virions (fd Mg3/pep23) to elicit a specific CTL activity against tumor cells. We have engineered fd Mg3/pep23 particles, which expressed the HLA-A2 restricted Mg3 epitope (FLWGPRALV), encompassing the 271-279 aminoacid sequence of the MAGE-3 tumor antigen, and the helper pep23 epitope (KDSWTVNDIQKLVGK) promiscuously recognized by several DR alleles. We have observed that APCs, pulsed with fd Mg3/pep23 phage particles, γ -irradiated, and incubated for 9-10 days with autologous HLA2⁺ PBMCs, induced a cytotoxic T lymphocyte (CTL) activity towards T2 (TAP-2 negative) target cells pulsed with the Mg3 peptide. We also observed that *in vitro* primed PBMCs were able to mediate a specific HLA-restricted cytotoxic activity towards HLA2⁺/MAGE-3⁺ tumor cell lines derived from human urinary bladder transitional cell carcinoma (TCC). Moreover, we have found that the administration of fd Mg3/pep23 phage particles to HLA-A2 (C57XSJL) transgenic mice induced specific CTL activity against RMA-S (TAP-2 negative) target cells pulsed with the Mg3 peptide. These results show the efficacy of double displaying bacteriophage fd virions (fd Mg3/pep23) to elicit a specific HLA-restricted CTL activity against tumor cells both *in vitro* and *in vivo*. Moreover, our data indicate that the Mg3 epitope is presented by urinary bladder carcinoma cell lines. An impaired presentation of this MAGE-3 epitope was described in melanoma cell lines; therefore, work is in progress to extend our study to a broad pattern of tumor target cells.

9. DENDRITIC CELLS GENERATED FROM HUMAN CD14⁺ MONOCYTES IN THE PRESENCE OF TYPE I IFN EFFICIENTLY STIMULATE AN EPSTEIN-BARR VIRUS-SPECIFIC CYTOTOXIC CD8⁺ T CELL RESPONSE

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Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus associated with several malignancies, including lymphoproliferative disorders in immunosuppressed individuals. Cell-mediated immunity, particularly the cytotoxic T lymphocyte (CTL) response, is considered of crucial importance for the control of EBV infection and EBV-associated lymphoproliferative diseases. Recently, dendritic cells (DCs) differentiated from human monocytes in the presence of GM-CSF and type I IFN (IFN-DCs) have been shown to rapidly acquire potent functional activities superior to those exerted by DCs differentiated with GM-CSF and IL-4 (IL-4-DCs) (Santini, *et al. J Exp Med* 2000;191:1777). In the present study, we evaluated the capability of IFN-DCs vs IL-4-DCs, pulsed with HLA class I-restricted EBV-derived peptides, to stimulate an EBV-specific CD8⁺ T cell response after co-cultivation with autologous PBLs. Higher frequencies of IFN- γ -producing T lymphocytes reactive against the selected EBV-derived peptides were detected by Elispot assays in PBL cultures stimulated with IFN-DCs as compared to IL-4-DC-stimulated cultures. Purified CD8⁺ T cells stimulated with peptide-pulsed IFN-DCs exerted significantly higher levels of cytotoxic activity against autologous EBV-infected lymphoblastoid cell lines as compared to IL-4-DC-stimulated counterparts. Interestingly, a more stable increase in the percentage of CD8⁺ cells with a memory phenotype (CD45RA⁻ CD27⁺) was detected in the cultures stimulated with peptide-pulsed IFN-DCs as compared to CD8⁺ cells co-cultured with peptide-pulsed IL-4-DCs. We then evaluated whether EBV peptide-pulsed IFN-DCs could expand *in vivo* specific CD8⁺ T lymphocytes capable of inhibiting lymphomagenesis in SCID mice reconstituted with PBMCs from an EBV-positive donor. Vaccination of reconstituted SCID mice with peptide-pulsed IFN-DCs resulted in a highly significant delay of lymphoma development as compared to what observed for unvaccinated mice and for mice vaccinated with unpulsed IFN-DCs. Overall, our results indicate that IFN-DCs can efficiently promote *in vitro* and *in vivo* the expansion of CD8⁺ T lymphocytes acting as cytotoxic effectors against EBV-transformed cells. This might lead to novel strategies of DC-based immunotherapy for EBV-associated and, possibly, other human malignancies.

10. VACCINATION OF HU-PBL-SCID MICE WITH HUMAN AUTOLOGOUS DENDRITIC CELLS PULSED WITH AT-2-INACTIVATED HIV-1 INDUCES NEUTRALIZING ANTIBODIES AND VIRUS-SPECIFIC CTLs AND CONFERS PROTECTION FROM HIV-1 CHALLENGE

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Even though HAART has markedly reduced mortality of HIV-infected patients and can diminish viral load to undetectable levels, a prompt viral rebound has been documented upon discontinuation of antiretroviral treatment. Unlike other human viruses establishing persistent infections such as EBV and CMV, HIV is not effectively controlled by the immune system. In fact, cytotoxic T lymphocytes are efficiently eluded by escape mutants and specific helper T cells may undergo HIV-induced cell death, while envelope glycoproteins generally fail to induce an efficient neutralizing antibody response. Current knowledge about HIV immunopathogenesis and recent data from animal models suggest that vaccine-induced immunity to HIV can be achieved under certain conditions and may represent an additional therapeutic option to be combined with HAART for the treatment of HIV-1-infected patients. Because of their prominent role of professional APCs, dendritic cells (DCs) are considered as ideal cellular adjuvants for the development of therapeutic vaccines for patients with certain malignancies or chronic infectious diseases.

We have recently demonstrated that peripheral blood monocytes can be induced to differentiate into highly active DCs (IFN-DCs) upon treatment with type I IFN and GM-CSF. Antigen-pulsed IFN-DCs exhibited a strong adjuvant activity and promoted a potent humoral and cellular Th-1 type response (*Blood* 2001;98:3022; *J Exp Med* 2000;191:1777). We have now found that both the humoral and cellular arms of immune response against HIV-1 can be induced *in vivo* by DC-based immunization of hu-PBL-SCID mice by using AT2-inactivated HIV-1 virions as immunogen. IFN-DCs proved to be superior with respect to DCs generated in the presence of IL-4 and GM-CSF in inducing a wide spectrum of anti-HIV-1 antibodies, including those directed to viral envelope glycoproteins, exhibiting a marked neutralizing activity against HIV-1. Immunization of hu-PBL-SCID mice with virus-pulsed autologous IFN-DCs resulted in the production of substantial levels of IFN- γ which was paralleled by the development of HIV-1-specific CD8 T cell responses. Finally, DC-based immunization of hu-PBL-SCID mice resulted in protection from the infection with HIV-1, as evidenced by the reduction of viral spreading to mouse organs. Altogether, these results suggest that IFN-DC-based immunization can represent a valuable strategy of therapeutic vaccination of HIV-1-infected patients to be associated with HAART with the aim of achieving a long-lasting control of virus replication.

11. DENDRITIC CELLS INDUCE THE DEATH OF HUMAN PAPILLOMAVIRUS TRANSFORMED KERATINOCYTES

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Although human papillomavirus (HPV) antigens are expressed in a majority of preneoplastic lesions (squamous intraepithelial lesions; SILs) of the uterine cervix, progression to invasive cancer may occur, suggesting that the presentation of viral antigens to the immune system is deficient in some SILs. To determine whether professional antigen-presenting cells die in SILs, we assayed for the apoptosis of immature dendritic cells (DC) in organotypic cultures of HPV-transformed keratinocytes which reproduce many features of *in vivo* observed SILs. Unexpectedly, the infiltration of organotypic cultures by DC specifically induced the apoptosis of HPV⁺ tumor cells whereas DC were not affected. In the same conditions and in co-cultures experiments, apoptosis was not observed in normal keratinocytes. The induction of apoptosis required membrane contacts between DC and HPV-transformed keratinocytes. Although the HPV⁺ cell lines were sensitive to the effects of TRAIL, soluble TRAILR2-Fc did not block the DC-induced apoptosis. Furthermore, although FasL and Fas were detected, on DC and HPV⁺ cell lines respectively, functional analysis revealed that this pathway is not responsible for the apoptosis induced by the DC. Altogether these results suggest that DC may be at the interface between innate and adaptive immunity by inducing the apoptosis of (pre)neoplastic cells.

12. DEFECTIVE DENDRITIC CELL MIGRATION IN PI3K γ ^{-/-} MICE IS ASSOCIATED WITH AN IMPAIRED T CELL SPECIFIC IMMUNE RESPONSE

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Dendritic cells (DC) are professional antigen presenting cells that play a pivotal role in specific immunity. Migration of maturing DC from peripheral tissues to secondary lymphoid organs is required for the initiation of specific immunity as well as for tolerance.

Chemokines are important chemotactic signals for several leukocyte populations including DC. Activation of chemokine receptors generates multiple signalling pathways. We have recently shown that activation of the gamma isoform of phosphoinositide 3-kinase (PI3K γ) is required for phagocyte migration both *in vitro* and *in vivo*. The aim of this study was to investigate the migratory ability of DC generated from PI3K γ ^{-/-} mice. *In vitro* CD34⁺-derived DC were cultured in the presence of GM-CSF and Flt3 ligand for 9 days (immature DC). Mature DC were obtained by adding TNF α for the last 24 hrs of culture. Immature DC from PI3K γ ^{-/-} mice displayed a strong reduction (40% \pm 9; n=5) of the chemotactic response to CCL3 and CCL5, compared with DC from wild type mice. Moreover, we observed the same degree of inhibition (39%) in migration of mature PI3K γ ^{-/-} DC to CCL19 compared with wild type DC. To evaluate the migration behaviour of DC *in vivo*, *in vitro* generated DC were fluorescence-labelled and subsequently injected in the foot pad of wild type mice. DC migration to lymph nodes was evaluated 24h after injection, by flow cytometry. *In vivo* migration of PI3K γ ^{-/-} mature DC was severely inhibited compared with wild type DC, with only 58% of the cells migrating to the lymph nodes (p<0.01). No differences in migration of wild type DC injected in wild type and PI3K γ ^{-/-} was seen, suggesting that the migratory impairment of PI3K γ ^{-/-} DC depend on the lack of enzyme, and not on the surrounding milieu. To distinguish if the migratory capacity involved only bone marrow precursor-derived DC, we studied cutaneous DC. Preliminary results showed an impaired emigration of cutaneous DC from skin explants into the culture medium in PI3K γ ^{-/-} mice compared with wild type. The addition of TNF α in the culture medium increased the emigration of wild type DC from explants. On the contrary, TNF α did not increase the emigration of DC from PI3K γ ^{-/-} skin explants (WT TNF α vs KO TNF α : p<0.005, n=10). *In vivo* FITC skin painting experiments confirmed these findings. Finally, to investigate the biological relevance of DC impaired migration in PI3K γ ^{-/-} mice, we performed *in vivo* experiment of delayed-type and contact hypersensitivity. Both animal model displayed a dramatic impairment in specific immune response in PI3K γ ^{-/-} mice compared with wild type mice, confirming the crucial role of this enzyme in the generation of a specific immune response.

13. INTERFERON- γ ACTIVATION OF INDOLEAMINE 2,3 DIOXYGENASE IN DENDRITIC CELLS CORRELATES WITH TOLEROGENIC ACTIVITY

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Indoleamine 2,3 dioxygenase (IDO) is the rate-limiting enzyme in the catabolism of tryptophan. The discovery that inhibition of IDO activity reduces the survival of MHC-mismatched fetuses in mice, and that the risk of fetal allograft rejection correlates with the degree of parental tissue incompatibility has led to the hypothesis that IDO activity protects fetal allografts from maternal T cell-mediated immunity. Our group has recently reported that murine dendritic cells (DC), in addition to macrophages, express high levels of IDO activity, which may account for the tolerogenic function of the CD8⁺ subset of splenic DC, particularly following activation by IFN- γ . We have hypothesised that the expression of IDO by DC, and its modulation by cytokine signals, may be a general mechanism required for the maintenance of T cell homeostasis and tolerance to self and tumor antigens. We demonstrated here that activation of IDO in CD8 α ⁺ DC is able to produce tryptophan catabolites, such as 3-hydroxyanthranilic and quinolinic acids, that are able to induce selective apoptosis of murine thymocytes and Th1 but not Th2 cells specific for a tumor/self antigen. T cell apoptosis was observed at relatively low concentrations of kynurenines and was associated with the functional activity of caspase-8. Moreover, *in vivo* treatment with 3-hydroxyanthranilic and quinolinic acid induced a selective decrease in double positive thymocytes population.

These data suggest that one mechanism accounting for the tolerogenic activity of IFN- γ treated DC involves IDO induction. Activation of this pathway regulates immunity by selectively deleting T cells and thus contributes not only to T cell homeostasis and self tolerance but also to immune deviation in disease states.

14. REGULATION OF LIFESPAN AND TURNOVER OF DENDRITIC CELLS BY TYPE I AND TYPE II INTERFERONS IN MICE

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Dendritic cells (DCs) can be activated by a variety of exogenous or endogenous stimuli. We have recently shown that type I IFN (IFN- α/β) is produced by dendritic cells and can act in an autocrine manner to activate DCs, by promoting their ability to prime naïve T cells (Montoya, *et al. Blood* 2002; in press). Type II IFN (IFN- γ) production by DCs is also upmodulated by IFN- α/β , indicating that IFN- γ could also play a role. In the present study, we have investigated on the role of IFN- α/β and IFN- γ in regulating the turnover and lifespan of DCs, by using knock-out mice for the respective receptors. We have found that DCs, especially the CD8 α^+ subset, from IFN- α/β R KO, but not IFN- γ R KO mice, display a reduced turnover rate, compared to WT CD8 α^+ DCs, as measured by BrdU incorporation in spleens. Moreover, injection of IFN- α/β (or Poly I:C) in normal mice determined an increase in BrdU uptake in CD8 α^+ DC subsets, indicating that IFN- α/β , but not IFN- γ , may play a role in regulating DC turnover *in vivo*. Interestingly, TUNEL assay and Annexin V/PI staining performed on purified splenic DCs after short-time culture indicate that IFN- α/β R KO DCs exhibit a higher survival rate compared to the WT counterparts. In addition, treatment with exogenous IFN- α/β of cultures of DCs obtained from normal mice increased the percentage of apoptotic cells, with respect to untreated DC cultures. These results suggest that IFN- α/β ability to modulate DC-survival may be responsible, at least in part, for its capacity to increase DC turnover *in vivo*.

15. IMPAIRED DIFFERENTIATION AND ACTIVITY OF DENDRITIC CELLS IN KNOCK-OUT MICE FOR ICSBP

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ICSBP is a transcription factor expressed exclusively in hematopoietic cells, which plays a critical role in regulating pathways affecting lineage commitment and myeloid cell differentiation. ICSBP-deficient (ICSBP^{-/-}) mice display high susceptibility to some infections, which has been correlated to the defective development and function of macrophages, as well as to a Th-2 polarization. Since dendritic cells (DCs) are crucial players in the initiation and regulation of the immune response, we have characterized the phenotype and activity of these cells in ICSBP^{-/-} mice. We have found that ICSBP^{-/-} mice have a markedly reduced number of the CD11c⁺ DC-subset expressing the CD8 α marker in spleen, lymph nodes and thymus. Freshly-isolated ICSBP^{-/-} CD11c⁺ CD8 α ⁺ DCs showed a less mature phenotype, expressing lower levels of co-simulatory molecules (ICAM-1, CD40, CD80, CD86). Moreover, these ICSBP^{-/-} DCs were not fully activated after short-time culture in presence with IFN- α/β , Poly (I:C), or LPS, as shown by phenotypic analysis and ability to stimulate an allogeneic proliferation in MLR assays, with respect to the WT counterparts. In addition, ICSBP^{-/-} mice displayed a significantly impaired number of the newly identified mouse plasmacytoid cells (pDC), the major producers of IFN- α/β , as revealed by marked reduction of CD11c^{low} Ly6C⁺ CD11b⁻ cells. Notably, purified splenic-DCs from ICSBP^{-/-} mice, expressed lower levels of IFN- α and IFN- β mRNAs and produced significantly reduced amounts of biological active IFN- α/β , upon *in vitro* stimulation with Poly (I:C) or LPS. Finally, ICSBP^{-/-} DCs expressed significantly lower amounts of IL-12p40 mRNA, while they exhibited higher levels of IL-4 and IFN- γ mRNA. Altogether, these results suggest a new role of ICSBP as a crucial factor controlling DC differentiation and function.

16. AMPLIFICATION OF TH1 IMMUNE RESPONSES BY IL-18. PATHOGENIC ROLE IN AUTOIMMUNITY

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The IFN gamma-inducing factor IL-18 has an important role in the activation and amplification of Th1-dependent immune responses, as well as in the stimulation of NK activity. The lupus-like autoimmune syndrome of MRL lpr/lpr mice is characterised by progressive lymphadenopathy and autoantibody production leading to early death for renal failure. It has been shown that IFN gamma is excessively produced by lpr/lpr cells and plays a pivotal role in abnormal cell activation and fatal development of the autoimmune disease. Since lpr/lpr cells are hyper-reactive to IL-18, deregulated IL-18 production and responsiveness could be among the causes of pathogenic IFN gamma production. In this study, the involvement of IL-18 has been examined in the progression of the lpr/lpr syndrome.

At variance with normal animals, autoimmune lpr/lpr mice hyper-express IL-18 in lymph nodes and other organs. To assess the contribution of IL-18 to the autoimmune pathogenesis, young lpr/lpr mice were vaccinated with a cDNA coding for the entire murine IL-18. Vaccinated mice produced autoantibodies to murine IL-18, and showed a significant decrease of autoimmune parameters (excessive IFN gamma production, spontaneous lymphoproliferation) and of the endorgan disease (glomerulonephritis and renal damage). Eventually, early mortality was significantly delayed and decreased in vaccinated mice.

It is concluded that stimulation of Th1 responses by IL-18 plays a major pathogenic role in the development of the autoimmune syndrome of lpr/lpr mice. Anti-IL-18 approaches could therefore be beneficial in the development of effective therapeutic strategies to autoimmune diseases, by inhibiting IL-18-dependent amplification of pathological Th1 responses.

17. A CRITICAL ROLE FOR LEPTIN IN DIFFERENT EXPERIMENTAL MODELS OF COLITIS

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Leptin, the product of the *ob* gene, regulates the balance of Th1/Th2 cytokines and modifies T cell immunity. Leptin-deficient *ob/ob* as well as leptin receptor (*Ob-Rb*)-deficient *db/db* mice are resistant against acute and chronic DSS-induced colitis, as evaluated by body weight, diarrhea, bleeding, histology, induction of proinflammatory cytokines, STAT-3 activation in the colon and rate of apoptosis in lamina propria lymphocytes (LPL). Similar resistance could be observed in the model of TNBS-induced colitis, in which reduced cytokine production and T cell activation associated with an increase of apoptosis in LPL was observed in *ob/ob* mice. *In vitro* studies revealed that leptin can directly induce STAT-3 activation in LPL and intraepithelial lymphocytes. To evaluate the role of T cells, we compared the effect of transferring CD4 CD45RB^{high} cells from WT and *db/db* mice into scid recipients. Initial experiments indicate that colitis induction by CD4 CD45RB^{high} cells from *db/db* mice is significantly reduced when compared to the transfer of WT cells, suggesting that leptin receptor expression on T cells is critical for development of intestinal inflammation. In conclusion, these results demonstrate that leptin represents a link between the endocrine and the immune system, which requires further investigations in experimental models and human IBD.

18. CORRELATION OF LOCAL IL-8 LEVELS WITH IgA AGAINST GARDNERELLA VAGINALIS CYTOLYSIN AND WITH MICROBIAL ENZYME ACTIVITIES IN WOMEN WITH BACTERIAL VAGINOSIS

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Bacterial vaginosis (BV) represents the main vaginal syndrome afflicting from 10 to 40% of fertile and pregnant women. In fact, prevalence of BV depends on race and social disadvantaged conditions. BV is a polymicrobial disorder characterized by an overgrowth of several anaerobic or facultative bacteria, mainly *Gardnerella vaginalis*, *Prevotella* spp., *Bacteroides* spp., *Mobiluncus* spp., and by a reduction or absence of lactobacilli colonization. Many basic questions regarding the pathogenesis of BV remain unanswered. Mucosal immune system activation may represent a critical determinant of adverse consequences associated with BV such as sexual HIV transmission, upper genital tract infections, post-surgical infections, and adverse pregnancy outcomes. Recently, a mucosal IgA response against the *Gardnerella vaginalis* cytolyisin (anti-Gvh IgA) was found in vaginal fluid of women with BV. Levels of sialidase, prolidase, anti-Gvh IgA were higher in vaginal fluid of 75 fertile women with BV compared to that of 85 healthy controls. Interleukin-8 levels were positively associated with anti-Gvh IgA response and inversely correlated with high levels of sialidase and prolidase activities in women with BV. Interleukin-8 was strongly associated with the number of leukocytes both in healthy and BV positive women. Absence of leukocytes in most women with BV is likely due to lack of interleukin-8 induction. Parallel impairment of innate and adaptive mucosal immune factors, likely through microbial hydrolytic effects, may allow the ascent of microorganisms to the upper genital tract, and may facilitate viral infections.

19. CYTOKINE EXPRESSION IN CIRCULATING T LYMPHOCYTES FROM PATIENTS UNDERGOING CAROTID ENDARTERECTOMY AND ITS RELATION TO THE MACROSCOPIC SURFACE APPEARANCE OF ATHEROSCLEROTIC PLAQUE

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Atherosclerotic plaques are heterogeneous vascular lesions. Changes in cell plaque composition are strictly related to the clinical outcome of the lesions. Although recent findings suggest a role of inflammation and cytokine regulation in the pathogenesis of atherosclerosis, the critical immunological and inflammatory mechanisms involved in plaque destabilization and rupture remain unknown. We investigated the possible association between immunological markers in whole peripheral blood (surface antigen and intracellular cytokine expression in T lymphocytes) and plaque morphology. Atherosclerotic plaques obtained from patients undergoing endarterectomy for carotid atherosclerosis were grouped according to the surface appearance of the lesions into two types (smooth and irregular) and four subtypes (calcific, fibrotic, fibrolipid and ulcerated). Blood samples were taken from 20 patients before surgery and from 8 healthy donors. Surface antigens (CD4, CD8, CD45RA, CD45RO, T cell receptor (TCR)- α/β , TCR γ/δ) and intracellular expression of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin-4 (IL-4) in CD4⁺ and CD8⁺ cells were determined by a three colour cytofluorimetric analysis. A higher percentage of T lymphocytes produced TNF- α and IL-4 in blood samples from patients than from normal subjects (TNF- α , 50% vs 20%; $p=0.01$). Intracellular expression of TNF- α , IFN- γ and IL-4 was higher in patients with irregular, i.e. advanced, unstable plaques than in patients with smooth, more stable plaques (TNF- α 55% vs 39%; $p<0.05$). No statistically significant differences were found in T cell phenotype characteristic among patients or between patients and controls. These results indicate a potential role of IFN- γ , IL-4 and predominantly TNF- α in circulating T lymphocytes as immunological markers of disease progression in patients with carotid atherosclerosis possibly leading to the development of immune modulatory tools that could halt progression.

20. DECOY RECEPTORS IN THE CHEMOKINE SYSTEM

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Decoy receptors are agonist-binding receptors that are not capable to transduce a signal. Unlike structural decoy receptors which present molecular features that render the signaling impossible (e.g. IL-1 type II receptor, IL-18R), functional decoy receptors are structurally able to transduce a signal but become uncoupled in some experimental conditions. We identified the experimental conditions leading to the generation of functional decoy receptors in the chemokine system. It is well established that Lipopolysaccharide (LPS) induces down-modulation of the surface expression of the chemokine receptors CCR1, CCR2 and CCR5. Monocytes and Dendritic Cells (DC), exposed concomitantly to LPS and Interleukin-10 (IL-10) for 24h, retain CCR1, CCR2 and CCR5 on the cell surface, but these receptors neither induce cell locomotion nor transduce signaling (Ca^{++} flux and PKB phosphorylation impaired). We confirmed and extended these results by investigating the phosphorylation of chemokine receptors induced by ligand binding. Monocytes treated with LPS+IL-10 showed hyper-phosphorylation of CCR5 compared to untreated and LPS- or IL-10- treated cells, and ligand binding did not induce further phosphorylation. Uncoupling of chemokine receptors occurred early after treatment (3-5h) and was partially reversed 24h after removal of the stimuli.

Comparison of the gene expression analysis (Affymetrix[®]) of DCs treated with LPS+IL-10 vs DCs treated with LPS or IL-10 alone indicated that the concomitant treatment (LPS+IL-10) induced or “super-induced” 88 genes after 2h, 80 after 8h and repressed or “super-repressed” 30 genes after 2h and 30 after 8h. The involvement of selected genes in the uncoupling of chemokine receptors will be studied in functional assays.

21. GENE EXPRESSION REGULATORY PROPERTIES OF CHEMOKINES: LEC/CCL16 INDUCES MONOCYTE CHEMOTACTIC PROTEIN-1 IN HUMAN MONOCYTES

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Liver-expressed chemokine (LEC) (CCL-16) is a human CC chemokine induced in human monocytes by treatment with LPS and IFN- γ , but its expression is dramatically increased in the presence of IL-10. LEC is a functional ligand for CCR1 and CCR8 and chemotactic for monocytes, dendritic cells and lymphocytes, but not for neutrophils. We and other authors have demonstrated that secretion of LEC by engineered TSA adenocarcinoma cells results in tumor rejection and prevents metastatic spread. Rejection is associated with an impressive recruitment of monocytes, lymphocytes and granulocytes that surround and infiltrate the tumor. The ability of TSA-LEC cells to promote this influx into the tumor seems even more remarkable when compared to the *in vitro* data on LEC chemotactic activity and suggests that high local concentrations of LEC induce downstream mediators that amplify leukocyte recruitment. On the basis of previous reports suggesting that chemokines modulate immune response by regulating gene expression, we have investigated the effect of LEC on chemokine and cytokine release by human monocytes. We show that LEC induces the expression of monocyte chemotactic protein-1 (MCP-1) and IL-8, but does not affect that of other chemokines such as RANTES, PARC and proinflammatory cytokines in human monocytes. Pre-treatment of monocytes with pertussis toxin and wortmannin, which respectively inhibit G-protein coupled receptors and PK3-kinase, prevents the MCP-1 induction by LEC, suggesting that its regulation on gene expression is mediated *via* G-protein coupled receptors. Since IL-10 upregulates LEC expression, we assessed its effect on the extent of MCP-1 production elicited by LEC treatment of monocytes. We observed IL-10 synergizes with LEC to induce MCP-1 expression and increases the chemotaxis of monocytes to suboptimal LEC concentrations. Thereby, we propose that LEC induces monocyte chemotaxis and activates MCP-1 expression in human monocytes, and that these activities are greatly potentiated by IL-10. The coordinated secretion of LEC and IL-10 may therefore enhance the extent of monocyte infiltration to the inflammatory site.

22. LOW PRODUCTION OF MDC BY MEMORY T CELLS AND IMPAIRED I-309 PRODUCTION BY RA SYNOVIAL T CELL; POSSIBLE EXPLANATION FOR THE DEFICIENCY OF TH2 AND REGULATORY T CELLS IN THE RHEUMATOID SYNOVIAL TISSUE

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The chemokines MDC and I-309 attract cells expressing CCR4 and CCR8, respectively. T helper (Th) 2 and regulatory T cells have been shown to express high levels of these chemokine receptors. Inflammatory Th1 cells dominate the T cell population in the synovial tissue (ST) in rheumatoid arthritis (RA) patients. Here, we analyse if low levels of Th2 and regulatory T cells in the ST could be the result of low production of MDC and I-309 by T cells in the ST. We observed a lower percentage of CCR4 and CCR8 positive lymphocytes in RA ST compared to peripheral blood. MDC was produced in significantly higher levels by naïve PB T cell lines compared to memory PB T cell lines and ST T cell lines in RA as measured by both ELISA and ELISPOT techniques. The same pattern was seen in naïve and memory PB T cells from healthy donors. MDC production was associated with the regulatory cytokine IL-10 on a clonal level. I-309 was secreted in similar levels by naïve and memory PB T cells in RA patients and healthy donors. However, I-309 was produced in significantly lower amounts by ST T cells than PB T cells in RA. The defect in MDC and I-309 by RA ST T cells might contribute to lack of regulation in the chronic inflammatory reactions in RA ST.

23. INFECTION OF HUMAN DENDRITIC CELLS WITH MYCOBACTERIUM TUBERCULOSIS INDUCES CYTOKINE AND CHEMOKINE GENE EXPRESSION THAT MODULATES T CELL RESPONSE

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Dendritic cells play an essential role in the initiation and maintenance of immune response to pathogens. To analyze early interactions between *Mycobacterium tuberculosis* (Mtb) and immune cells, monocyte-derived dendritic cells (MDDC) were infected with Mtb. The internalization of Mtb resulted in the maturation of MDDC as reflected by enhanced expression of several surface antigens. Moreover, the production of IFN-gamma-inducing cytokines, IL-12 and IFN-alpha was observed in Mtb-infected MDDC. In line with these findings, supernatants from Mtb-infected MDDC induced IFN-gamma production by T cells and enhanced IL-18 receptor expression. Neutralization of IFN-alpha and IL-12 activity in Mtb-infected MDDC supernatants suggested that IL-12 and, to a lesser extent, IFN-alpha play a significant role in enhancing IFN-gamma synthesis by T cells.

Interestingly, we also observed in Mtb-infected MDDC the synthesis of CXCL10, a CXC chemokine produced in response to IFNs and microbial products. We observed that the synthesis of CXCL10 was in part dependent on Mtb-induced IFN-alpha/beta production, as neutralization of IFN- alpha/beta reduced the production of this chemokine. Chemotaxis assays performed using supernatants of Mtb-infected MDDC, clearly demonstrated a chemotactic activity for activated CD4, and CD8 cells expressing CXCR3 receptor.

Altogether, our results suggest that MDDC are engaged in inducing T cells following Mtb infection in virtue of the expression of costimulatory molecules, the production of Th1/IFN-gamma-inducing cytokines and chemokines involved in the recruitment and selective homing of activated/effector cells, which are known to accumulate at the site of infection and to form the granulomas. Thus, the development of a new generation of vaccine against tuberculosis has to elicit a strong activation of MDDC in order to stimulate the maximal antigen presentation, the production of IFN-alpha, IL-12 and CXCL10 and consequently a protective T cell response.

24. IL-16 IN THE INDUCTION OF THE HUMAN CD34⁺ HEMATOPOIETIC CELLS INTO DENDRITIC CELLS: IN VITRO

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To address the question of whether IL-16 has a role other than as chemotactic and activating factor for CD4⁺ cells, and whether it induces CD34⁺ cell population to proliferate and/or differentiate versus dendritic cells (DCs), we examined the capability of human CD34⁺ progenitor cells to mature into DCs in the presence of IL-16. CD34⁺ cells were cultured in the presence of GM-CSF, IL-4, Flt-3ligand, TNF- α , in standard conditions, plus either SCF (control) or IL-16. After 7, 11 and 14 days of culture, the obtained cells were compared with regard to fold increase of nucleated cells number, morphologic features and surface phenotype. Mixed leukocyte reactions (MLRs) were performed to assess the allostimulatory activity of DCs, and the proliferative response of allogeneic T cells was evaluated by flow cytometry as bromodeoxyuridine (BrdU) incorporation by CD3⁺ cells. Our results indicate that IL-16 does induce differentiation of CD34⁺ precursors into DCs. IL-16 exerts this function more efficiently than SCF: total nucleated cells were increased 56.1 (range 35.4-71.9), or 32.4 (range 21.0-45.3) respectively, compared with the initial number of CD34⁺ cells at the time of starting culture. The efficiency of IL-16 is particularly evident after 7 days of culture, when out of the total nucleated cells, 80.5% \pm 3.4% (mean \pm S.D.) of the cells express CD1a in the presence of this cytokine, compared with the percentage obtained in the presence of the other factor (SCF: 62.8 \pm 6.6). The CD1a⁺ cells express CD4, HLA class I and II molecules, and the costimulatory molecules CD83, CD80, CD86 and CD40. In allogenic MLRs, cells in the presence of IL-16 showed potent antigen presenting capacity similar to DCs obtained in the presence of SCF. Our results suggest a new role of IL-16, i.e. induction of the CD34⁺ hematopoietic progenitor cells to develop into functional and mature DCs. In addition to showing the potential involvement of IL-16 in normal hematopoiesis *in vivo*, this work also provides a feasible culture technique to rapidly obtain human dendritic cells in sufficiently large amounts to evaluate their role in transplantation immunity and to explore potential therapeutic uses.

25. CYTOKINES AND INTERLEUKIN-DEPENDENT CURSOR CLONE IN HEMATOPOIETIC DIFFERENTIATION

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Interleukin-3(IL-3)-dependent progenitor clone LyD9 and its interleukin-4(IL-4)-dependent derivative clone K-4 were shown to differentiate into myeloid cells as well as B lymphocytes by coculture with bone marrow stroma cells. The establishment of IL-3-dependent progenitor cell lines, each of which can differentiate into either B or T lymphocytes, represents a model system for molecular biological studies on cellular commitment. Recently, the IL-3 dependent LyD9 was also shown to differentiate into B cells when cocultured with bone marrow stroma cells and T cells.

Unpublished:

1-In the present study, we employed stem cells from CD34⁺/THY1⁺ blood cells of three patients with Hodgkin's disease surviving 22, 28, 32 years. The progenitor cells were transfected by electroporation with a plasmid coding a mutant enzyme dihydrofolate reductase.

2-We employed lymphoid-stroma cells isolated from the lymphonode of a patient with malignant lymphoma and characterised by a monoclonal antibody against the T-zone stroma cells in long term culture. Here, we demonstrated that the IL-3-dependent progenitor clone (LyD9) and its IL-4-dependent derivative (K-4) have capacities to differentiate into myeloid as well as B-lymphocytes when cocultured with human peripheral blood CD34⁺/THY1⁺ cells containing a high number of CDW90- Thy-1 population highly enriched for cells of long term cultures.

Phenotypic changes in the intermediate clone K-4 will provide a clue to understand molecular events required for differentiation of early precursor cells in the bone marrow and in blood. It remains to be determined whether LyD9 cells or stroma cells produce IL-4 during the coculture. An important and helpful point from the study is that acquisition of capacity to respond to different growth factors is associated with differentiation of the LyD9 clone into B-lymphocyte and myeloid cells. It is possible to test the results into expression of specific receptors and to induce differentiation into a particular lineage when cDNAs of various receptors are available.

26. DEVELOPING OF GENE NETWORK AND MATHEMATICAL MODEL OF MACROPHAGE ACTIVATION BY INTERFERON-GAMMA AND LIPOPOLYSACCHARIDES

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Activated macrophages perform various immunological functions in organism using influence of different substances, whose synthesis is regulated by macrophage activation gene network. The gene network is a group of coordinately expressing genes that control the maintenance of different functions in organism. The group of genes form nucleus of the gene network; moreover, gene network involves RNA and proteins, encoded by these genes, nonproteinaceous substances and relationships.

Since macrophage functions disbalance was observed in some of pathologies, the process of macrophage activation is being studied actively. Large amount of experimentally established facts has now accumulated. It is known that gene network of macrophage activation includes synthesis of several cytokines, cellular receptors, nonproteinaceous substance and that some feedback effects also exist. However, complete picture of macrophage activation is still not clarified. Moreover, depending on the activator type, diverse ways of signal transduction work and hence diverse sets of genes are activated.

Our aim was development of united scheme of macrophage activation gene network and accumulation of corresponding information in GeneNet system. An accent was made for interferon-gamma and lipopolysaccharides activation. Development of mathematical model of this gene network is partially finished and some useful results are obtained.

Gene network scheme is available *via* the Internet (<http://www.mgs.bionet.nsc.ru/systems/MGL/GeneNet/>). Gene network database is regularly updated; this version contains information about approximately 200 different gene network components including more than 50 proteins, 27 genes and more than 100 elementary events.

The developed mathematical model describes the process of macrophage activation in terms of chemical kinetics. It allows to predict some quantitative changes of macrophage activation process, to foresee some properties of synthetic substances (acting on macrophage) and can also indicate presence of failing link or substance on the scheme. As well, it allows to study behavior of gene network in different states of functioning.

The developed gene network and corresponding mathematical model could be useful for solving a wide range of biologic and medical problems without performing expensive experiments.

27. QUANTITATIVE AND QUALITATIVE SIMULTANEOUS ANALYSIS OF PROTEINS FROM A SINGLE DROP OF SAMPLE

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Cytokines and kinases play pivotal roles in cell signaling events, mediating a wide range of physiological responses including immunity, inflammation and hematopoiesis. They are also associated with a spectrum of diseases ranging from tumor growth to infection to Parkinson disease. Here, we present data from the Bio-Plex Protein Array System demonstrating the sensitive, simultaneous quantitation of 18 cytokines in human or mouse sera, and in tissue culture media. We can quantify all 18 cytokines at a limit of detection less than 10 pg/ml, and with inter- and intra-assay CV less than 10%. Using an 18-plex assay, a single 96-well plate can generate 1,728 concordant cytokine values from 12 μ l of sample/well. We also demonstrate a multiplexed sandwich immunoassay that simultaneously measures phosphorylation levels of JNK, p38MAPK, Erk, Akt, and IkBa from a cell extract in a single well of a 96-well plate. These high content, multiplexed assays are highly correlated with and as sensitive as ELISA and immunoblotting. All the assays are specific and exhibit no detectable cross-reactivity between antibodies. Most importantly, data obtained from multiplexed cytokine and phosphoprotein assays provide an invaluable profile of cellular response for drug discovery and development.

28. INTERFERON- γ ELISPOT ASSAYS ON MULTISCREEN PLATES

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The ELISPOT (Enzyme Linked Immuno-Spot) assay provides an effective method of measuring the antibody or cytokine production of immune cells on the single cell level. The popularity of this assay has seen a resurgence in recent years as researchers attempt to gain a better understanding of immune responses in a variety of applications. The test is frequently used instead of standard ELISA assays of serum, because the lower limits of detection using ELISPOT can be many times more sensitive than with serial dilution ELISA assays. Millipore provides efficient tools for this kind of assay in a 96 well plate format, thanks to our patented MultiscreenTM 96 well membrane bottomed plate. The MultiscreenTM 96 well membrane bottomed plate consists of a 96 well plate with a filter membrane sealed on the bottom of every well and with a support underdrain mounted on the bottom of the plate itself. The underdrain is used in order to prevent either cross-contaminations among the wells and to keep the membrane integrity during the experiment. The underdrain is being removed only at the end of the detection step, in order to allow the membrane removal and the evaluation of the results. For ELISPOT assays, we tested two different kind of filter membranes: the Hydrophobic Polyvinylidene Fluoride (PVDF) ones which are on the Multiscreen-IP plates, and the Mixed Cellulose Esters (MCE) ones which are on the Multiscreen-HA plates. The following protocol is an example of a typical Elispot assay for the production of interferon-gamma. The results gave a comparison between the two types of membranes, in terms of sensitivity, background and protein binding. It may be modified as necessary for other compounds and applications.

29. REGULATORY B CELLS CAN PREVENT THE DEVELOPMENT OF ARTHRITIS

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The interaction of CD40 expressed on B cell and CD154 on T cell has been shown to play an important role in T cell dependent activation of B cells. In this study we show that anti-CD40 mAb activation of arthritogenic splenocytes give rise to a B cell population producing high levels of IL-10 and low levels of IFN γ . Transfer of this B cells into newly immunized DBA/1-TcR-b-Tg mice inhibited Th1 differentiation and arthritis development. Here we have also shown that interleukin (IL)-10, but not IL-4, is essential for the generation of this B cell population. Co-treatment with an anti-IL-10 receptor (IL-10R) monoclonal antibody abrogated inhibition of arthritis mediated by the anti-CD40 mAb challenged B cells, suggesting that the anti-CD40 stimulation, by upregulating IL-10 production, drives the generation of a subset of B cells, named B regulatory cells. Finally, we have also observed a mild, although significant delayed of disease onset of arthritis in mice recipient of B cells isolated from isotype treated control splenocytes. This finding suggest that regulatory B cells are a normal component of the splenocytic pool and that their regulatory action can be amplified through CD40 stimulation.

30. THE BINDING SUBUNIT OF PERTUSSIS TOXIN UNCOUPLES T CELL ACTIVATION AND PROLIFERATION FROM HIV REPLICATION IN LYMPHOID TISSUES

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It has been previously shown that pertussis toxin (PTX) inhibits SIV replication in infected monkeys, HIV replication in *in vitro* infected PBMC and virus expression from chronically infected promonocytic U1 cells. Of note, the anti-HIV effect of PTX has been reproduced by its non toxic, binding subunit, B-oligomer (PTX-B) that also showed inhibitory effect on macrophage infection. Due to the importance of lymphoid tissues for HIV replication, we investigated the antiviral effect of PTX-B in these compartments.

Inhibition of both R5 and X4 HIV-1 replication was consistently observed and inversely correlated to the dose of PTX-B: 80% inhibition was obtained at 10 pM and reaching no effect at dose of 5 nM. Concomitantly, PTX-B exerted a mitogenic effect on both naive (CD45⁺/CD62L⁺) and activated (CD25⁺/CD69⁺/HLA-DR⁺) CD4⁺ and CD8⁺ T cells. CD4⁺ T cells were infected but, surprisingly enough, did not support HIV replication unless they were stimulated *in vitro* with PHA+IL-2. In contrast, PTX-B restimulation did not induce virus production.

Thus, PTX-B potentiates T cell activation and proliferation concomitantly with the inhibition of HIV replication. Similar results were obtained by using a compound retaining all the PTX-B properties, PT-9K/129G, already used as an *in vivo* vaccine against Bordetella Pertussis infection. Therefore, PTX-B or the PT-9K/129G represent a novel class of potential immunostimulatory agents also endowed with antiviral effects against HIV.

31. IMPROVEMENT OF IL-2 PRODUCTION AND RESTORATION OF STROMAL CELL FUNCTION IN HIV-1 PATIENTS AFTER HAART

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Background: Patients infected by HIV-1 may present with a variety of different hematological abnormalities in addition to a progressive deficiency of immune system. Increasing evidences indicate that BM suppression results from viral infection of accessory cells, with impaired stromal function and alteration of hematopoietic growth factors network.

Objectives: We have investigated the effects of HAART on cytokine and chemokine production by BM cells, including stromal cells, in a group of subjects with HIV-1 infection.

Methods: BM cytokine production has been evaluated by ELISA, stromal cells were studied by immunohistochemistry and PCR analysis for HIV-1 DNA.

Results: Compared with uninfected controls, an altered cytokine and chemokine production by BM cells was observed, characterised by decreased IL-2 and elevated TNF- α , MIP-1 α , MIP-1 β , and RANTES levels, along with a defective BM clonogenic activity. This pattern was partially reverted by HAART. An increased BM clonogenic capability was observed in association with normalisation of IL-2 production and modifications of chemokine receptors expression on CD34⁺ cells. The BM accessory cells before HAART were represented by macrophage-like cells, in some cases positive for HIV-1 DNA, suggesting that these cells are the main target of HIV-1 infection. During HAART, the stromal cells became predominantly fibroblastoid-like, as observed in normal controls, and resulted negative for HIV-1 DNA.

Conclusions: Controlling HIV-1 replication by HAART may determine an amelioration of stem cells activity, a restoration of stromal cell pattern and functions, with increased IL-2 production also at BM level.

32. INCREASED CD4 AND CCR5 EXPRESSION AND HIV-1 ENTRY IN CD40L-STIMULATED MACROPHAGES

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The effects of a soluble trimeric CD40L agonist has been investigated on the expression of CD4 and CCR5, as well as on human immunodeficiency virus type-1 (HIV-1) entry and replication in human macrophages. CD40L increased the number of CD4 and CCR5 antibody binding sites (ABS) and the percentage CD4- and CCR5-expressing cells. Infection of CD40L-stimulated macrophages with HIV-1 resulted in a marked increase of viral DNA with respect to controls, as demonstrated by PCR assay. HIV-1 p24 antigen analysis showed that peak viral production was not different between CD40L-stimulated macrophages and controls. However, due to a prolonged life span, overall viral output was increased in CD40L-stimulated cultures. Finally, CD40L downregulated the antiviral efficacy of compounds that inhibit HIV-1 reverse transcriptase. In conclusion, CD40L-stimulation of macrophages can contribute to plasma viral load and favour the establishment of a pool of latently infected macrophages that can be reactivated to release virus.

33. EXPRESSION OF IMMUNOMODULATING MOLECULES ON THE SURFACE OF THE VACCINE VECTOR STREPTOCOCCUS GORDONII

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Streptococcus gordonii, a commensal of the human oral cavity, has been developed as a vaccine delivery vector. The genetic system allows the integration of heterologous genes into the resident chromosome and expression of recombinant proteins at the bacterial surface. Recombinant streptococci expressing vaccine antigens were shown to be capable of inducing both systemic and local immune responses following parenteral and/or mucosal immunization in different animal models (mice and monkeys). Recently, the genetic system has been improved by simultaneously expressing two different proteins on the same bacterial cell. With this strategy, it is possible to co-express a vaccine antigen in combination with immunomodulating molecules and adjuvants. We have co-expressed the model vaccine antigen tetanus toxin fragment C (TTFC) with two immunomodulating molecules: the CTA1-DD fusion protein and the B subunit of the heat-labile toxin of *Escherichia coli* (LTB). CTA1-DD consists of the enzymatically active cholera toxin A1 subunit fused to a dimer of the Ig-binding D-region of *Staphylococcus aureus* protein A responsible for targeting B cells. Co-expression of TTFC with CTA1-DD or LTB on the surface of *S. gordonii* was confirmed by cytofluorimetric analysis and Western blot. In order to assess the adjuvant effect of these molecules, C57BL/6 and BALB/c mice were immunized by the intragastric route with *S. gordonii* expressing TTFC alone or in combination with either CTA1-DD or LTB. In C57BL/6 and BALB/c mice, levels of TTFC-specific serum IgG doubled using recombinant bacteria co-expressing CTA1-DD, and antigen-specific IgA were also increased in feces only in C57BL/6. The adjuvant effect of LTB was observed only in BALB/c mice. In this case, 46% of mice developed high concentrations of TTFC-specific serum antibodies (100-1000 folds higher than mice immunized with recombinant bacteria expressing TTFC alone). A local IgA increase was not detected, although analysis of bacterial persistence in the intestinal mucosa showed a higher percentage of colonised BALB/c compared to C57BL/6 mice. These results show the potential of bacteria to be used for expression of multiple heterologous proteins, making them an integral part of the search for new methods of developing combined vaccines.

34. USE OF MF-59 AS ADJUVANT IN A VACCINE FOR EQUINE FLU

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Equine flu is a very infectious respiratory disease, that generally runs its short course unless there are complications like bacterial infections that prolong the animal's recovery. In the horses-racing world, especially for strong blood-line horses, the commercial value is very high, therefore the flu represents a large economic burden for the owner.

The flu viruses type A/H7N7/Prague/1/56 and A/H3N8/Miami/1/63 are more commonly found in horses and they are known as viruses 1 and 2. The virus 1 circulates broadly throughout the world and, from 1956 to now, there have been minor variations. From 1972 also virus 2 had undergone antigenic variations that have made two new types: A/H3N8/Newmarket/1/93 and A/H3N8/Newmarket/2/93, that are in the formulation of the vaccine used in this study. It is evident the need to find new vaccines or make the existing ones better as the commercial vaccines do not work very well. One way of trying to fix the problem is by mixing the vaccine with an adjuvant which stimulates the immune system response and elongates its duration.

In this study, a commercial sub-unit vaccine is compared with the same vaccine that has the addition of MF-59, a oil-water emulsion, already utilized for human use. The principal objective of this work was to verify if the use of the adjuvant increases the immunogenicity in respect to the traditional vaccine.

We have used two groups of 7 horses each, one with the traditional vaccine and the other with the vaccine that has the addition of MF-59. We have done the dosage of antibodies before and after the vaccination with the method SRH. From the obtained results, we can see that the use of adjuvant has produced a higher protection level above all for A/H3N8/Newmarket/1/93 and A/H3N8/Newmarket/2/93. The GMT increased for all three antigens from time T0 to time T1. Concerning the reactogenicity the adjuvant-vaccine has the same results as the traditional. For horses, the future possibilities for finding new kind of vaccine are interesting and exciting.

35. TUMOR-DERIVED HEAT-SHOCK PROTEIN GP96 AND CYTOKINES AS VACCINE THERAPY IN MELANOMA PATIENTS

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Although tumor-derived HSP-gp96 induces T cell-mediated protection in mice models, no direct evidence of peptide presence in gp96 molecules has yet been reported in human tumors. Using antigen-specific CD8⁺ T cells, we were able to demonstrate that gp96 purified from melanoma cells does contain peptides derived from tumor antigens, such as MART-1 (27-35). To assess the immunological and clinical effects of HSP, we performed a phase I-II clinical trial based on vaccination with autologous tumor-derived HSP-gp96 in stage IV melanoma patients. Eleven out of 23 vaccinated patients displayed a statistically significant increase of HLA-class I-mediated IFN γ T cell secretion in response to either autologous or allogeneic tumor cells, as assessed by Elispot. A strong increment of MART-1(27-35) recognition, detected by Elispot and HLA/peptide tetramer staining, was also observed in 3 of the 12 HLA-A2⁺ melanoma patients. Two of them responded clinically to the vaccine with a complete tumor regression. Immunohistochemical analysis of melanoma lesions used for gp96 purification showed a statistically significant association between HLA-class I expression and clinical/immunological responses to the vaccine. In fact, none of the patients bearing HLA-class I negative melanoma displayed changes in T cell-mediated anti-tumor activity or signs of tumor regression. Altogether, these data suggest that melanoma-derived gp96 represents a promising tool for vaccine therapy. However, immunological and clinical efficacy of such immunization protocol need to be improved. To this aim, IFN- α for increasing HLA-class I expression in melanoma cells and local GM-CSF for DC recruitment at vaccine site will be included in the treatment schedule. A randomized trial for assessing the ability of such cytokines to improve gp96-based vaccine efficacy in stage IV melanoma patients is currently ongoing in our Institution.

36. PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF DC GENERATED WITH GM-CSF AND INTERFERON- α : POTENTIAL ROLE IN ADJUVANT DC-BASED VACCINE FOR MELANOMA PATIENTS

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In order to boost anti-tumor immune responses in melanoma patients submitted to radical lymphadenectomy and to reduce their risk of recurrence, we designed a clinical protocol aimed at evaluating the immunological role of adjuvant vaccine therapy based on DC and tumor lysate in such patients. The protocol, based on the usage of autologous dendritic cells (DC) loaded with tumor lysate derived from allogeneic melanoma cell lines, is designed to potentially generate polyclonal anti-melanoma T cell responses directed against a large tumor antigenic repertoire, including molecules recognized by anti-tumor CD4⁺ T cells. Based on recent results about the role of IFN- α in the differentiation and activation of DC (Santini, *et al. J Exp Med* 2000;191:1777), we decided to evaluate the possibility of generating DC from peripheral blood by *in vitro* culture with IFN- α and GM-CSF. Monocytes-enriched PBMC from HLA-A2⁺ healthy donors were cultured for 3 days in the presence of GM-CSF (50 ng/ml) and different types and doses of IFN- α . DC were then recovered and evaluated for the expression of different phenotypic markers and for their ability to generate antigen-specific T cells after *in vitro* stimulation. Among the different IFN- α types tested, 'natural' IFN- α (by Alfa Wasserman) at 10E4 U/ml mediated the best results, allowing the generation of CD14⁻, CD1a⁺ DC with activated phenotype and expressing high levels of costimulatory molecules such as CD40, CD80, CD86 and HLA-DR. These DC were also able to readily generate from HLA-A2⁺ PBMC specific anti-Flu, MART-1 and HBV or EBV T cell responses. On the basis of these results, we are currently investigating the possibility of raising tumor-specific T cells by stimulation with melanoma lysate-pulsed DC with the aim of standardizing GMP-like culture conditions and melanoma-lysate loading protocol.

37. INTERFERON- α PROMOTES THE RAPID DIFFERENTIATION OF MONOCYTES FROM PATIENTS WITH CHRONIC MYELOID LEUKEMIA INTO ACTIVATED DENDRITIC CELLS EXPRESSING TH-1-PROMOTING FACTORS AND COMPETENT FOR THE INDUCTION OF AUTOLOGOUS CD8⁺ T CELL EXPANSION

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Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disease arising from the clonal expansion of an altered stem cell and associated with the expression of the bcr/abl oncogene. An historical advance in the treatment of patients with CML has been achieved by the clinical use of interferon(IFN)- α , which has represented the first line therapy for many years. However, the overall mechanisms of action of IFN are still unclear. Recently, several reports have underscored the potential importance of IFN- α effects on the host immune system for the generation of a long-lasting antitumor response in CML patients. These findings appear to be consistent with a recent evidence suggesting that alterations in the function of antigen presenting cells (APCs), especially dendritic cells (DCs), may be involved in disease pathogenesis and progression. In the present study, we have evaluated the role of IFN- α in the differentiation and functional activity of DCs from CML patients. For these studies, we used monocytes from patients at diagnosis of CML, as at this stage of the disease the large majority of cells carry the oncogenic bcr/abl fusion protein and may express other CML tumor-associated antigens (TAA). We found that treatment of CML monocytes with IFN- α /GM-CSF resulted in the rapid generation of partially mature DCs, expressing important immunoregulatory molecules such as IL-15 and IP-10, and competent to induce proliferation of autologous T lymphocytes and expansion of CD8⁺ T cells. Notably, the immature DCs generated in the presence of IL-4/GM-CSF required a further exposure to LPS in order to achieve similar characteristics, in terms of cytokine/chemokine production and stimulation/expansion of T cells. Altogether, these results support the concept that effects of IFN- α on DC differentiation/activation can be important for the clinical response of CML patients after IFN- α treatment and suggest that the use of this cytokine can be advantageous for the rapid generation of highly active DCs for vaccination strategies of CML patients.

38. ANTI-CANCER VACCINATION USING AS A SOURCE OF ANTIGENS *IN VIVO* DYING TUMOR CELLS

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During apoptotic cell death, intracellular antigens are cleaved, modified and clustered in membrane blebs, thus becoming available to antigen presenting cells. Antigen-specific T cells and non-MHC restricted unconventional cytotoxic cells commit tumor cells to apoptosis. During the killing, granzyme B cleaves intracellular antigens in fragments not generated during any other form of cell death, revealing cryptic epitopes (Casciola-Rosen, *et al. J Exp Med* 1999;190:815). Granzyme-killed tumor cells may represent therefore a unique source of TAA. In this study we focused on the possibility to trigger an efficient anti-tumour immune response against the B16 murine melanoma, using as source of TAA tumor cells *in vivo* killed by IL-2 activated cytotoxic cells (CTLs). To this aim we *in vitro* derived IL-2 activated CTLs, culturing spleen-derived T cells for 3 days in complete medium supplemented with 1000 U/ml rh-IL-2. IL-2 activated CTLs express low amounts of CD3, CD8 (but not CD4), NK1.1 and IL-2 receptors, as detected by FACS analysis. They efficiently killed both NK-specific targets (like YAC-1 leukaemia cells) than B16 melanoma cells. They did not kill syngeneic blasts nor immature DCs. The life span of CTLs upon intra-tumor injection is being evaluated, as well as the ability of the elicited tumor apoptosis to productively activate (prime) bona fide MHC-class I-restricted α/β T cells, by means of tetramer staining and cytotoxicity assays. We are also verifying whether a protective immune response ensues, endowed with memory and specificity. We are in parallel evaluating if the presence of professional antigen presenting cells (dendritic cells, DCs) into the tumor parenchyma further influences the outcome of tumor killing *in vivo*.

39. VIRAL LOAD AND CELLULAR IMMUNE RESPONSE IN PATIENTS WITH CERVICAL HPV-ASSOCIATED PRENEOPLASTIC LESIONS

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Background: Cellular immune defects have been observed in patients with human papillomavirus (HPV)-associated lesions of the uterine cervix. These data suggest that the restoration of a cellular immune response could be beneficial for these patients. We have therefore established a vaccination protocol with a mutated E7 protein of HPV16 in patients with low grade cervical lesion. The goal of this study was to set up protocols to follow up the HPV16 viral load and the systemic immune response against E7 during the clinical trial.

Methods: Real time PCR was chosen to quantify the viral DNA on cervical brush specimens whereas the immune response against HPV is evaluated using an IFN γ intracellular staining on PBMC stimulated *in vitro* with the vaccine protein. To set up these techniques, cervical specimens and blood of patients with low grade and high grade lesions were used.

Results: In our group of 13 patients, 7 were HPV positive by classical PCR. We performed real time PCR on HPV⁺ patients using HPV16 specific primers and probe (Wieland, *et al. J Invest Dermatol* 2000;115:396). In these HPV⁺ patients, 4 were positive for HPV16 with one with very high amount of HPV16 copies.

We performed IFN γ intracellular staining on PBMC of patients and normal donors after an overnight stimulation. As positive control, cells were activated with PHA and for all patients and normal donors we observed cells producing IFN γ . Surprisingly, the percentage of IFN γ ⁺ cells seemed to be higher in HPV16⁺ patients. After stimulation with mutated E7 protein, we observed a response against this protein in some HPV⁺ patients with the highest response in the patient showing the highest copy number of HPV16.

Conclusion: We have developed techniques allowing to determine the HPV16 viral load and the immune response against HPV 16 during the vaccination protocol.

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