Instituto Juan March
de Estudios e Investigaciones

156 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Dendritic Cells: Biology and Therapeutic Applications

Organized by

R. M. Steinman, I. Melero and A. L. Corbí

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C. Ardavín
L. Chen
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The lectures summarized in this publication were presented by their authors at a workshop held on the 6th through the 8th of October, 2003, at the Instituto Juan March.

Depósito legal: M- 47844 / 2003
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid

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Introduction

Ralph M. Steinman, Ignacio Melero and Angel L. Corbí

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Dendritic cells are bone marrow-derived cells which play a pivotal role in the generation and control of natural and adaptive immunity. As professional antigen presenting cells, dendritic cells display a very high capacity to stimulate naïve T lymphocytes for the generation of primary immune responses, promoting the generation of distinct types of effector T cells which ultimately will lead to the initiation of immune responses or the induction of tolerance. Besides, dendritic cells are now recognized as main players in innate immunity, as they are capable of recognizing numerous pathogen-associated molecular patterns, and secreting cytokines which mobilize and activate macrophages and natural killer cells. Their ability to fulfil both activities probably derives from the existence of various dendritic cell subsets, all of which are included within the "dendritic cell" system that controls immunity. Research on dendritic cells has greatly expanded in the last years because of their potential application to modulate immune responses with therapeutic purposes. In this regard, and because of their functional plasticity, dendritic cells could be seen as "tools" not only to implement cell-based immunotherapies for cancer, but also to down-modulate undesired immune responses in autoimmune diseases. Based on all these ideas, numerous clinical trials are currently underway on a variety of malignancies, and results will be known in the coming years.

The aim of this meeting has been to discuss the generation of the distinct dendritic cell subsets and their precursors, their role in the generation of immune responses, T cell polarization and immune tolerance, and the feasibility of the therapeutical applications of dendritic cells. The importance of polarizing cytokines, co-stimulatory molecules and pathogen-recognition receptors on the initiation of immunity by dendritic cells was addressed in depth, and results were presented revealing the existence of functional interactions between dendritic cells with NK and regulatory T cells. The meeting also allowed ample discussion on the different approaches taken for dendritic cell-based cancer immunotherapy, and for comparison of all the currently available information and some preliminary data from ongoing trials. In this regard, some basic consensus concepts were reached which might serve as starting points for future clinical trials. As a whole, the workshop has also proven very fruitful for the exchange of ideas between laboratories involved in basic and clinically-oriented research on dendritic cells.

The organisers,
October 2003

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Session 1: Ontogeny, differentiation and maturation of dendritic cells
Chair: Ralph M. Steinman

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Dendritic cells, antiviral immunity and autoimmunity

Rolf M. Zinkernagel

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Immunity i.e. specific resistance against infectious diseases is based on crucial innate or natural resistance (e.g. interferons) plus antibody and T cell mediated specific immunity. While survival of new-born and young hosts against infections crucially depends on specific immunity, this is not efficient against later diseases including tumours. Immunity may cause autoimmunity which will contribute to disease sometimes after 30. It is interesting to note that antibodies (also in bird, fish and amphibian eggs) contribute crucially to immunity and are the basis of all efficient vaccines but on the other hand they contribute also importantly to autoimmunity.

Experimental evidence attributes to secondary lymphatic organs a key role in handling and storing antigens to drive both, antibody and T cell mediated immunity. IgM antibodies are often T help independent and are short lived, they therefore do not cause autoimmunity. In contrast switch to IgG depends upon $CD4^{+}$ T help. IgG is long-lived and therefore can be associated with autoimmunity. An example is shown, where induction of cytotoxic T cells against a strictly peripheral self antigen expressed in pancreatic β islet cells will cause an acute diabetes type I, whereas prolonged immunisation will cause a B cell destruction that is associated with an auto-antibody response and a chronic inflammation resulting in neo-formation of lymphatic tissue in the islets (see also Hashimoto thyroiditis). Thus sufficient antigen has to be transported to the draining lymph-node or spleen for a limited time to induce a response, or alternatively, a lymphatic tissue is exported to peripheral solid inflamed organs (involving various chemokines). The autoimmune response is then maintained and only halts once autoantigen is completely eliminated. A similar scenario can be shown for peripheral solid tumours such as sarcomas and carcinoms. Tumour cells themselves reaching draining lymph-nodes or spleen early at low numbers will induce a cytotoxic T cell response directly without a need for cross-presentation or cross-priming. Once induced, such effector T cells may eliminate tumour cells but only if the number of activated effector cells over time is sufficient to eliminate small solid tumours in the periphery rapidly enough before mutants escape. This can be enhanced by introducing into the peripheral tumour 'so called' second signals that seem to be helpful in maintaining effector T cell function extra-lymphatically for a prolonged time.

In our hands using spleen cells expressing viral antigens or tumour cells expressing various viral antigens we cannot demonstrate efficient cross presentation and cross-priming of cellular antigens to $CD8^{+}$ T cells. The role of cross-priming was also evaluated in viral infections. We used polio virus (PV) that cannot productively infect mice in the absence of a

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transgenic human PV(R) receptor. Nevertheless, normal mice can respond with a CD8$^+$ T cell response in absence of a PVR if live but not if formaldehyde fixed or UV-irradiated PV is used for immunisation. These results indicate that some viral genes seem to function after uptake of PV in absence of a receptor and that sufficient translation accurs to properly present peptides via class I MHC on host cells for induction of a CD8$^+$ T cell response. Therefore MHC class I loading is at least much more efficient via intracellularely synthesised sources than from extracellular materials. Very special tricks have to be used to overcome this physiological barrier. Several groups have shown that high concentration of antigen and introduction of corpuscular virus like particles containing antigen may overcome that limitation, particular if mixed with CpG. Nevertheless under physiological viral infection conditions or in tumour situations cross-presentation/cross-priming is not efficient enough to induce a protective or controlling cytotoxic T cell response. Coevolution has avoided early death of too many hosts by viruses or tumours before successful reproduction.

We conclude that the function of dendritic cells is mainly to transport and process antigens in a non-replicating form to reach MHC class II pathways or by infection (or some tricks including membrane fusions) to reach the cytosole and be presented also via MHC class I. The role of so called second signals on dendritic cells seems much less important compared to the necessity for antigen to reach secondary lymphatic organs. In contrast so called second signals 'in trans' amplify T cell responses and are helpful in maintaining an immune response in peripheral solid tissues including auto-immune targeted organs (undesired) or solid peripheral tumours (desired).

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Multilineage origin of dendritic cells

Carlos Ardavín

Dendritic cells (DCs) are key components of the immune system owing to their essential role in the induction and control of T cell immunity, as well as in the modulation of B cell and NK cell responses. Research on DCs over the last decade has led to the concept that the DC system comprises a large collection of subpopulations with different functions. Functional specialization allows defined DC subsets to induce efficient defence mechanisms against pathogens and tumor cells, and maintain T cell tolerance (reviewed in Banchereau & Steinman 1998). A key question, with important implications for our understanding of the induction and control of immunity by DCs, as well as the use of DCs for immunotherapy, is whether DC functional diversity results from the existence of developmentally independent DC subpopulations, or whether DC subsets sharing a common differentiation origin acquire specific functions in response to environmental signals (reviewed in Ardavín 2003). Although a definitive model of DC development remains to be established, recent data derived mostly from *in vivo* DC reconstitution assays in the mouse indicate that the same DC subpopulations, including $CD8^-$ DCs, $CD8^+$ DCs and $B220^+$ plasmacytoid DCs, can be derived from either myeloid or lymphoid progenitors (Martín et al. 2000, Traver et al. 2000; D'Amico & Wu, 2003). Therefore these experiments do not support the existence of independent myeloid and lymphoid DC subpopulations as previously proposed, but a DC differentiation model relying on a dual contribution of myeloid and lymphoid differentiation pathways. On the other hand, recent data from our laboratory indicate that during microbial infections, blood-borne DC precursors are recruited to the reactive sites where they differentiate into fully competent DCs, including including $CD8^-$ DCs, $CD8^+$ DCs and $B220^+$ plasmacytoid DCs (Martínez del Hoyo et al. 2002), suggesting the possibility that all DC subsets derive from a single DC common progenitor.

The analysis of DC-specific transcription factors and genes that are related to DC differentiation and function, as well as the study of the differential involvement of DC subpopulations in *in vivo* immune responses against pathogens and tumor cells should provide important insights into the present controversy regarding both the developmental origin of DCs, and the functional correlation between DC subpopulations.

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Intracellular signaling and transcription during dendritic cell differentiation and maturation

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The unsurpassed efficiency of Dendritic cells (DC) in antigen-presentation is acquired only after they have completed a differentiation programme commonly referred to as "maturation". DC maturation, far from being unique and "one-way" process, appears to be pathogen-specific and exhibits huge quantitative and qualitative stimulus-dependent variations (1). We have previously reported that expression of the CD49d integrin subunit is greatly upregulated in monocyte-derived DC (MDDC) in response to a variety of maturation agents, thus endowing mature DC with increased capacity for cell-extracellular matrix and cell-cell interactions (2). Dissection of the molecular basis for integrin expression in MDDC revealed that CD49d upregulation is differentially influenced by MER-ERK and p38MAPK activation. Moreover, both signaling pathways exerted opposite effects on LPS- and TNFα-induced MDDC maturation and IL-12 p70 production (3), suggesting that the relative levels of activation of ERK and p38MAPK in maturing DC might modulate the polarization of naive T cells. Concomitant analysis of gene expression profiles in maturing DC has now allowed the identification of Runx3 (AML-2) as a factor whose transcription increases upon DC maturation, as well as the identification of a new Runx3 isoform specifically induced upon LPS- or TNFα-induced DC maturation. Runx3 has been subsequently found to control the expression of adhesion molecules essentially implicated in the stablishment of the imunological synapse (6).

On the other hand, the process of differentiation of monocytes into DC is driven through the combined action of GM-CSF and IL-4. To determine the signaling pathways and transcription factors driving the monocyte-DC transition, we have foccussed on DC-SIGN (4), a dendritic cell-specific lectin that functions as a pathogen-associated molecular pattern (PAMP) receptor. DC-SIGN expression was found to be IL-4 dependent, and its induction was inhibited in the presence of IFN, TGFβ and anti-inflammatory agents (5). The IL-4-dependency of DC-SIGN induction correlates with the presence of functional STAT6-binding sites in the proximal regulatory region of the DC-SIGN gene. In fact, blockade of STAT6 activation prevents IL-4-induced DC-SIGN expression in monocytes and in certain myeloid cells. Results on the the signaling requirements for DC-SIGN expression, and evidences about the signaling ability of DC-SIGN, will be presented.

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Toll-like receptors and control of mammalian immunity

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Toll-like receptors (TLRs) comprise a family of pattern-recognition receptors that detects conserved pathogen-associated molecular patterns (PAMPs). These are products produced uniquely by microorganisms but not by host organisms. TLRs function as crucial sensors of microbial infection. Upon recognition of microbial products, TLRs induce signaling pathways that initiate inflammatory and immune responses. The signaling pathways activated by individual TLRs are complex, and it is becoming evident that different TLRs can trigger differential signaling, thereby enabling the host to tailor the immune response to the infectious agent.

In addition to directly triggering innate host defense responses, TLR-mediated recognition and signaling plays a crucial role in regulating adaptive immune responses. While the details of these controls are only starting to emerge, there appears to be at least two distinct pathways of TLR-mediated control of T cell activation. One has to do with the co-stimulatory pathway, and the other with the cytokine-dependent block of regulatory T cells (Tr cells). TLR-induced cytokines, in particular IL-6, render pathogen specific T cells refractory to the suppressive effect of Tr cells, thus allowing induction of protective pathogen-specific T cell responses.

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Identification of a myeloid intrathymic pathway of dendritic cell development marked by expression of the GM-CSFR.

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In this study, the finding that a significant proportion of all dendritic cells (DCs) resident in vivo in the human postnatal thymus displayed a myeloid-related phenotype prompted us to re-examine the developmental origin of thymic DCs, a cell type hitherto considered to represent a homogeneous lymphoid-derived population. We show here that these novel intrathymic DCs are truly myeloid, as they arise from CD34+ early thymic progenitors through CD34lo intermediates which have lost the capacity to generate T cells, but display myelomonocytic differentiation potential. We also demonstrate that phenotypically and functionally equivalent myeloid precursors devoid of T cell potential do exist in vivo in the postnatal thymus. Moreover, although IL-7 supports the generation of such myeloid intermediates, we show that their developmental branching from the main intrathymic T cell pathway is linked to the upregulation of the myelomonocytic GM-CSF receptor, to the downregulation of the IL-7 receptor and to the lack of pre-TCRa (pTa) gene transcriptional activation. Taken together, these data challenge the current view that the thymus is colonized by a lymphoid-restricted progenitor and provide evidence that a more immature precursor population with lymphoid and myelomonocytic potential is actually seeding the human postnatal thymus.

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Session 2: Antigen recognition, adhesive and migratory capabilities of dendritic cells
Chair: Francisco Sánchez-Madrid

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Phagoplasmic reticulosomes

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Induction of cytotoxic T cell immunity by dendritic cells requires the phagocytosis of pathogens, virus-infected or dead tumour cells. After phagocytosis, antigens are exported from phagosomes into the cytosol and degraded by the proteasome. The resulting peptides are thought to be translocated into the lumen of the endoplasmic reticulum (ER) by specific transporters associated with antigen presentation (TAP), and loaded onto MHC class I molecules by a complex "loading machinery" (which includes tapasin, calreticulin and Erp57). The process of presentation of peptides derived from phagocytosed antigens to on major histocompatibility complex (MHC) class I molecules CD8+ T lymphocytes, is called "cross presentation". We have shown that dendritic cells have developped a specific pathway for antigen export to the cytosol, which is selective for the size of antigens and uniquely found in cross presenting cells. We have also shown that soon after or during formation, phagosomes fuse with the ER. This fusion event results in the recruitment of the complete ER MHC class I-loading machinery into phagosomes (including TAP, tapasin, calreticulin, erp57). After antigen export to the cytosol (through yet unknown pathways) and antigen degradation by the proteasome, peptides are translocated by TAP into the lumen of the same phagosome, before loading on phagosomal MHC class I molecules. Therefore, cross-presentation in dendritic cells occurs in a specialized, self-sufficient, ER–phagosome mix compartment: the phagoplasmic reticulusomes.

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DC-lectins recognition of self and non-self

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Dendritic cells (DC) are specialized in the recognition of pathogens and play a pivotal role in the control of immunity. They are present in essentially every organ and tissue where they operate at the interface of innate and acquired immunity by sensing pathogens at the periphery and presenting pathogenic peptides to draining lymph node residing T cells. Because of their antigen-presentation capacity and their immune stimulatory function, dendritic cells are attractive cells to use in anti-cancer therapy. C-type lectin receptors expressed by DC are involved in the recognition and capture pathogens. To date seven different C-type lectins have been identified on DC. It is now becoming clear that these C-type lectin receptors may not only serve as antigen receptor recognizing pathogens to allow internalisation and processing to enhance antigen presentation, but may also function as adhesion molecules and signaling molecules. We have demonstrated that the DC-specific C-type lectin DC-SIGN functions as an antigen-receptor as well as an adhesion receptor on DC. As pathogen receptor DC-SIGN recognizes the envelope protein gp120 of HIV-1 and the cell wall component ManLam of Mycobacteria. Targeting of these pathogens to DC-SIGN however leads to immune escape as HIV-1 captured by DC-SIGN escapes the DC-SIGN antigen presentation route, hiding as infectious particle in non-lysosomal compartments, and Mycobacterium tuberculosis manipulates the activation machinery of Dendritic cells.

To date little is know on the specificity by which C-type lectins interact with self-glycoproteins and pathogens. Conserved sequences (CRD) in the C-type lectin domain recognize unique carbohydrate (mannose) structures that can be specifically expressed by certain pathogens as well as self-proteins. Detailed analysis of the carbohydrate recognition profile of DC-SIGN revealed novel carbohydrate specificity and concomitantly novel pathogens and tumor antigens that express these structures and interact with DC-SIGN on DC. Some pathogens can escape immunity through binding of C-type lectins, by affecting either intracellular trafficking or intracellular signaling properties of C-type lectins by affecting TLR signaling. The finding that especially C-type lectins recognize carbohydrate structures on pathogens opens up a new area of research that studies the potency of C-type lectins to interact with distinct glycosylated tumor antigens. This will facilitate tumor antigen internalisation into the DC and subsequent enhanced antigen presentation by DC which would benefit optimal use of DC in anti-cancer therapy. Understanding the diversity of C-type lectins being expressed on DC as well as their carbohydrate specific recognition profile will be instrumental to understand DC pathogen recognition in many pathogenic disorders, as well as the regulation of cellular interactions of DC which are essential in the control of immunity.

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High and low affinity saccharide ligands for murine SIGN-R1 revealed by carbohydrate array and cell binding approaches

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Members of the carbohydrate-binding 'C-type' lectin family have emerged as key players on endothelia at the initial stages of leukocyte recruitment in inflammation, and as immuno-modulators on antigen-presenting cells. Knowledge of details of the carbohydrate ligands for these receptors is crucial for understanding the molecular basis of cell-cell interactions as well as cell-pathogen interactions in innate and acquired immunity.

However, the pinpointing of oligosaccharide ligands remains a challenging area of cell biology. This is because ligands that are oligosaccharides cannot be readily cloned each being the product of multiple glycosyltransferases. A carbohydrate microarray system recently established for generating large repertoires of immobilized oligosaccharide probes is proving to be a powerful means both of detecting protein-carbohydrate interactions and assigning the sequences recognized (1). This communication is concerned with elucidation of the carbohydrate ligands for murine SIGN-R1 (a molecule related to the dendritic cell receptor, DC-SIGN), which is expressed on marginal zone macrophages and is known to mediate the uptake of polysaccharides such as dextran (2,3). Probing oligosaccharide microarrays with the recombinant soluble SIGN-R1 has revealed a range of blood group Lewisa- and Lewisx-related fuco-oligosaccharides and also high-mannose type N-glycans as ligands. Dextran binding is detectable only with the cell-associated protein. Cell binding and inhibition experiments with mannosyl and dextran probes, and monosaccharides, indicate that mannosyl/fucosyl glycans are high affinity ligands and dextran is a low affinity ligand for SIGN-R1. These findings open the way to elucidating the immunological sequelae of cell binding and uptake of structurally distinct carbohydrate ligands mediated by this protein.

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Interaction of the tumor antigen Muc1 with C-type lectins on dendritic cells

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Muc1 is a high molecular weight glycoprotein, normally expressed on the apical surface of ductal epithelial cells and some leukocyte subsets. It is over-expressed and aberrantly glycosilated in several adenocarcinomas, such as breast cancer, making it an interesting target for immunotherapy (1). Dendritic cells (DCs) have the unique capacity to initiate antigen specific immune responses. They express several uptake receptors, allowing recognition of a broad range of antigens. Among these receptors are C-type lectins which recognize glycan structures expressed on self tissue and pathogens. The role of several C-type lectins remains to be elucidated (2), but they may be crucial for initiation of immune responsesto glycosilated antigens, such as Muc1.

Our aim was to identify C-type lectins on DCs involved in Muc1 recognition and to study whether tumor associated Muc1 is differently recognized than normal Muc1. We used recombinant Muc1-Fc fusion proteins with defined glycosilation pattern, often expressed by tumor associated Muc1 (T and Tn antigens) and studied their interaction with monocyte derived DC. DC bound Muc1-Tn better than Muc1-T, in a $Ca2+$ dependent manner, whereas they only marginally bound Muc1 bearing sialylated T antigens. We studied several lectin transfectants and identified the Macrophage Gal/GalNAc specific Lectin (MGL) as a receptor for Muc1. Interestingly, MGL did not recognize normal Muc1 but only tumor related Muc1. We are in the process of analysing Muc1 uptake, intracellular routing and presentation by DC. Analyzing the factors and conditions involved in Muc1 antigen presentation by DCs may provide the basis for an optimal DC-based immunotherapy against breast cancer.

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Innate control of DC activation

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Dendritic cells (DC) play a major role in the initiation and regulation of adaptive immune responses and, therefore, the manipulation and/or targeting of DC has tremendous potential for cancer immunotherapy and for vaccination against cancer-promoting infectious agents. However, these clinical applications are hampered by the fact that we still understand relatively little about how DC become "activated" to stimulate and direct T cell responses. We have been studying three distinct "pattern-recognition" pathways that may trigger murine DC activation by potential pathogens. One pathway involves DC recognition of, among others, extracts of *Toxoplasma gondii*, bacterial DNA and mycobacterial fractions. This recognition is mediated by distinct toll-like receptors (TLRs) and one of its hallmarks is the ability to trigger synthesis of high levels of the Th1-promoting cytokine, IL-12. Another pathway involves recognition of yeasts by a cell surface receptor and leads to production of another cytokine, IL-10, via a kinase not previously implicated in pattern recognition. Finally, a third pathway involves cytosolic recognition of viral double-stranded RNA via protein kinase R and promotes secretion of high levels of type I interferons, another group of immunomodulatory cytokines. In each case, cytokine synthesis can be markedly amplified by signals subsequently provided by newly-activated $CD4^{+}$ T cells. The cytokines may then instruct those T cells to adopt different effector fates. Thus, these data suggest distinct ways in which DC could be manipulated to favour different types of immune responses.

DC are a heterogeneous family and understanding whether DC subsets perform distinct functions in the immune system is also critical for clinical applications. We have used microarray analysis and other methods to identify several gene products that can be used to distinguish murine DC subsets. For example, we have found that murine DC subsets express distinct TLR repertoires but that they differ from their human counterparts in that plasmacytoid and non-plasmacytoid DC do not display a dichotomy in TLR9 and TLR4 expression. At the same time, we have been examining the behaviour of purified murine DC subsets in a range of functional assays *in vitro*. Among our most notable findings, is the observation that the production of hallmark cytokines such as IL-12, IL-10 or IFNα by DC subsets is not dictated by ontogeny but by pattern recognition. This is best exemplified by the observation that appropriate viral signals can switch conventional DC into high interferon producers, a property previously thought to be restricted to the plasmacytoid DC subset. Similarly, we have shown that microbial signals can override any intrinsic Th directing capacity, allowing all subsets tested to date to prime Th1 or Th2 responses *in vitro*. We hope that these and further studies will lead to the development of new strategies to target and activate DC, thereby improving their use as tools for immunotherapy in cancer and infectious disease.

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Monocyte subsets, differentiation to dendritic cells, and the role of CCR8

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Monocytes represent a potential source of dendritic cells (DCs), but in contrast to other DC subsets, monocytes are not committed to differentiate to DCs and apparently indeed more often become macrophages *in vivo*. Because learning to manipulate the differentiative fate of monocytes in situ may enhance immunotherapeutic approaches, we have pursued mechanisms that explain how monocytes divert to DCs. The use of IL-4 and GM-CSF, while immensely useful for generating DCs from monocytes, may not be the optimal model to study differentiation because it appears to fully divert differentiation to DCs. We predict that additional new information on the regulation of monocyte fate can be studied by tracing monocytes in a setting that supports both macrophage and DC differentiation.

Thus, an *in vitro* model of a simple tissue was constructed to study this problem using human monocytes. In this model, some monocytes can become migratory DCs, while other develop into macrophages in the same cultures. Recently, we have found that heterogeneity within the circulating population of monocytes is influential to determining fate, and that particularly a subset of monocytes that express CD16 (FcγRIII) preferentially becomes migratory DCs over the more dominant CD16- subset. Relative to CD16- monocytes, the CD16+ population of human monocytes has been recently shown to express higher levels of CX3CR1, HLA-DR, HLA-DP, and CD86, whereas they express lower CCR2, CD62L, CD14, CD36, and CD64 when directly analyzed after purification from blood. In various disease states, including HIV, cancer, and rheumatoid arthritis, the blood manifests an elevation in the frequency of the CD16+ monocytes. There is some evidence that CD16- monocytes may be the precursors of CD16+ monocytes, but the conditions that promote the development of these subsets *in vivo* is not known.

Representing an advance in tracing monocytes in mouse, corresponding subsets of mouse monocytes have been identified (Geissmann, Jung, and Littman; Immunity 19:71-82, 2003). They differentially express Gr-1. Gr-1^{hi} mouse monocytes share patterns of chemokine receptor and adhesion molecule (especially CD62L) expression with CD16- human monocytes, and Gr-1^{lo} mouse monocytes share these features with CD16+ human monocytes. In mice, it has been shown that the two subsets possess distinct trafficking patterns, and it is further argued that the populations may not be related as a precursor-product pair.

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In wild-type mice, we observed that Gr-1^{lo}, but not Gr-1,^{hi} monocytes express CD86, CD40, I-A^{b}, and CD8α, which is in keeping with their resemblance to $CD16^{+}$ human monocytes. The blood of most mice showed the presence of Gr-1^{int} monocytes, and these cells expressed an otherwise intermediate phenotype between Gr-1^{hi} and Gr-1^{lo} monocytes, in keeping with the possibility these cells were intermediates in a continuum in transition from one stage (Gr-1^{hi}) to the other (Gr-1^{lo}). Gr-1^{lo} monocytes were apparently not recruited to the peritoneal cavity after injection of thioglycollate, as previously reported, but nevertheless, Gr-1^{int} and Gr-1^{hi} monocytes were both found in the peritoneal lavage. A fraction of these peritoneal cells readily developed into DCs after 2 days culture in GM-CSF.

CCR8 is scarcely studied, but is known to be expressed by human monocyte-derived cells, regulatory T cells, Th_2 cells, and NK cells. It is also the target of some viruses that produce CCR8 antagonists, although the immunological benefit, if any, gained by the virus for such targeting is not known. We have observed that Gr-1^{lo}, but not Gr-1^{hi}, mouse monocytes are greatly reduced in frequency in the blood of CCR8 deficient mice. Correspondingly, there is a reduced number of migratory DCs found in CCR8-/- lymph nodes after injection of latex particles in skin to label incoming monocytes. Further analysis indicated that the reduced recovery was not due to overall reductions in monocyte recruitment, but rather to a relative failure of Gr-1int monocytes to appear at the site of recruitment. CCR8+/+ peritoneal monocytes much more efficiently converted to DCs compared with CCR8-/- monocytes. We sorted Gr-1^{int} and Gr-1^{hi} peritoneal monocytes and cultured them separately in GM-CSF. In 2 days of GM-CSF culture, only the sorted Gr-1^{int} cells began to express CD11c, and these cells were more stimulatory to mouse T cells in an MLR. Neutralizing mAb to CCL1, a confirmed ligand for CCR8, partially blocked the differentiation of Gr-1^{int} monocytes to DCs, such that allostimulatory capacity was reduced by half. These data illustrate that Gr-1^{int} monocytes can rapidly develop into DCs and that this development is supported by signals from CCR8.

Next we were led to test whether CCR8 participated in differentiation and migration of human monocytes. Indeed, neutralizing mAb to CCR8 blocked the appearance of migratory DCs in the model of transendothelial trafficking. This could be due to either effects on differentiation and/or migration. Support for the former possibility was obtained by examining the transition of CD16- monocytes to CD16+ cells in the presence of TGFβ1. This step was blocked by anti-CCR8 mAb, in agreement with the data obtained in mice. Screening of populations of human skin DCs for expression of CCR8 revealed that this molecule may be particularly relevant to monocyte differentiation and migration, since CCR8 was expressed only on CD14+ emigrants from skin, and not likely broadly relevant to the control of Langerhans cell migration, for instance.

Overall, these data lend insight into the control of the monocyte differentiation and argue that the CD16+ human monocyte/Gr-1^{lo} mouse monocyte stage of differentiation particularly supports development of DCs. We argue that more analysis is required to

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determine whether Gr-1^{hi} monocytes are a lineage distinct from Gr-1^{lo} monocytes, and that the two populations may be related. These data furthermore reveal a novel function for CCR8 in regulating differentiation of monocytes, apparently impacting differentiation directly, thereby likely only indirectly affecting migration.

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Session 3: Dendritic cell instruction of T lymphocyte differentiation
Chair: Carlos Ardavín

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Dendritic cells and the control of immunity and tolerance

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A newly appreciated dimension to dendritic cells (DCs) is their role in peripheral tolerance. Several pathways to tolerance have become apparent in different experimental models: deletion, anergy, Tr1 type regulatory cells and CD4+ CD25+ suppressors. Each can take place when DCs are presenting antigen in vivo in the steady state, i.e., in the absence of overt inflammation or infection. Underlying the function of DCs in tolerance are two broad sets of activities. One is their tissue distribution and movement. For example in the steady state, DCs are found within and beneath epithelial surfaces, where they can capture dying cells and environmental proteins, migrate to the T cell areas of lymphoid tissues, and gain access to the recirculating pool of lymphocytes. A second set of activities involves the handling of antigens. DCs express a number of receptors that can enhance antigen uptake and in turn, processing and presentation of peptides on both MHC class I and II products. We will discuss these points with observations on the model antigen, ovalbumin, given to mice by different routes and forms: as a soluble protein, as a surrogate antigen within dying cells, or as a conjugate linked to an antibody to the DEC-205 endocytic receptor on most DCs in lymphoid organs. In each case, presentation takes place on MHC class I and II, the former by an efficient exogenous pathway requiring TAP transporters. In the steady state, the exogenous pathway leads to tolerance of specific CD8+ T cells primarily by deletion.

Most DCs in the steady state, including those expressing the DEC-205 endocytic receptor in the T cell areas, are immature. These DCs have the capacity to take up, process and present antigens but do not induce immunity, i.e., the production of effector T cells and memory. For immunity to develop, it is necessary to administer a stimulus that leads to DC maturation. In lymphoid tissues, DC maturation is manifest by many changes, including the upregulation of several costimulatory molecules, like CD40/80/86, and the production of cytokines like TNFα, IFN-γ and IL-12, but relatively modest increases in the activation of antigen specific T cells. Several stimuli for DC maturation are known from in vitro studies, e.g., TNF family molecules especially CD40L, and ligands for Toll Like Receptors. We have studied two maturation stimuli in vivo, agonistic anti-CD40 monoclonal antibodies and the synthetic glycolipid α-galactosyl ceramide. Both agents convert the outcome of DC antigen presentation from tolerance to immunity. Interestingly, when antigen is targeted to maturing DCs in vivo, the immune responses can be much greater than those observed with current gold standards, such as antigen in complete Freund's adjuvant and antigen-pulsed ex vivo derived DCs.

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This dual role in immunity and tolerance helps DCs solve a challenging problem. During infection, as will be illustrated with influenza, DCs capture a mix of antigens derived from the microbe as well as dying self tissues and the environment. Therefore, while carrying out their protective roles, maturing DCs need to avoid the danger of priming for autoimmunity and chronic inflammation. This can be alleviated beforehand through efficient antigen uptake and processing pathways followed by tolerance. The function of DCs in tolerance and immunity should help to identify antigen-specific mechanisms for disease pathogenesis and protection.

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Regulation of T cell immunity by dendritic cells

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Immune responses are initiated in the T cell areas of secondary lymphoid organs, where naive T lymphocytes encounter dendritic cells (DCs) that present antigens taken up in peripheral tissues or locally. Antigens that do not have access to DCs are generally ignored by T cells, while those that do have access to DCs can stimulate naive T cells, driving their proliferation and differentiation or, in special circumstances, their deletion. Thus, DCs represent the interface between the universe of foreign and tissue-specific antigens and T lymphocytes and are the key players in the regulation of cell-mediated immunity. I will address two main points: i) how DC can provide under steady state or inflammatory conditions widely different levels of T cell stimulation leading to either tolerance or immunity; ii) how the overall strength of T cell stimulation determines T cell proliferation, survival and differentiation to effector and memory T cells. Strategies to increase the efficacy of DC based vaccination and adoptive T cell therapy will be discussed.

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Analysis of a novel costimulatory molecule B7-DC

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DCs play a pivotal role in innate and adaptive immune responses as professional APC. To stimulate and activate naïve T cells both signal through TCR engaging peptide-MHC (signal 1) and signal through ligands of costimulatory molecules (signal 2) are required. With only signal 1 but not signal 2, T cells become anergic. The molecular complexity of costimulation is demonstrated by the glowing number of B7 family members, namely B7-1, B7-2, and the recently discovered ICOSL, B7-H1/PD-L1, B7-H3. A novel costimulatory molecule, B7-DC (PD-L2), another member of the B7 family, has been cloned. B7-DC has high homology to B7-H1 and although both bind PD-1, their expression patterns are very different. B7-H1 is expressed on hematopoetic cells, nonhematopoietic tissues and tumors, but B7-DC expression is restricted to DCs.

B7-DC has been shown to be costimulatory by us, while others have demonstrated an inhibitory role. We have found a synergistic effect between B7-DC and B7-1/2 for CD4+ T cells in proliferation and cytokine production in vitro. This was due to faster expression kinetics of CD40L on T cells stimulated with B7-DC than on T cells stimulated by B7-1, and synergistic expression of CD40L by B7-DC and B7-1. Moreover, we generated B7-DC gene deficient (B7-DC KO) mouse, and demonstrated that DCs from B7-DC KO mice are diminished in their ability to induce proliferation of naïve antigen specific CD4+ T cells, which do not express PD-1. On the other hand, DCs from B7-DC KO mice have similar or slightly better ability to induce proliferation of activated antigen specific CD4+ T cells, which express PD-1, demonstrating that B7-DC serves predominantly a stimulatory function in the initiation of CD4+ T cell responses.

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VLA-4 is at the pSMAC of the immunological synapse and deviates a Th1 response *in vivo* and *in vitro*

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The integrin VLA-4 (alpha4beta1), not only mediates adhesion and transmigration of leukocytes, but also provides costimulatory signals to the TCR/CD3 complex and induces T lymphocyte activation. However, the behavior of VLA-4 during the formation of the immune synapse between a T lymphocyte and an APC or the immune consequence of its costimulatory signals during antigen presentation have not been studied before. Here we show that VLA-4 is recruited to both a human and murine antigen-dependent immune synapses, either when the APC is a B cell or a dendritic cell (DC), co-localizing with LFA-1 in the pSMAC ring. Antibody engagement of certain epitopes of a4 integrin triggers a Th1 response in a human in vitro model of naïve T cell priming by DC. The administration of alpha4 antibodies to healthy Brown Norway (BN) rats also induces an immune deviation to Th1 response, and reverts the balance of Th1/Th2 polarization in a model of Th2 autoimmune nephritis in BN rats. Together, these data reveal an unexpected regulatory role of alpha4 integrins on T-APC cognate immune interactions.

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Dynamics of adhesion molecules involved in DC-T lymphocyte immune synapses

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The physical interaction between DC and T lymphocyte is the first step to initiate the primary immune response. Exploratory adhesive interactions of T lymphocytes and APCs are essential for the specific recognition of antigenic peptides before the formation of a immune synapse. We have reported the role of T lymphocyte ICAM-3 in T cell-APC conjugate formation prior to antigen recognition, in early intracellular signalling and in cytoskeletal rearrangement. On the other hand, although the behavior of T lymphocyte receptors during the establishment of the immune synapse has been widely studied, less is known about the participation of DC receptors in conjugate formation. We have determined the dynamic behaviour of DC adhesion receptors, (ICAM-3, ICAM-1, LFA-1) as well as antigen recognition receptors (MHC-II) during the establishment of immunological synapses. Videomicroscopy studies with DC transiently transfected with GFP constructs revealed that ICAM-3, ICAM-1 are concentrated at the trailing edge and translocate to the contact area during DC-T lymphocyte recognition. At initial time of interaction, ICAM-3-GFP formed discrete transient clusters; subsequently, LFA-1-GFP, ICAM-1-GFP and MHC-II-GFP were similarly recruited to the IS. After 20 min, all of these receptors were stably concentrated to the IS. In contrast, we found that other molecules, such as CD44-GFP, do not redistribute to the DC contact area with the T cell. Three-dimensional analysis showed that ICAM-1, ICAM-3 and LFA-1 are organized in a peripheral adhesion ring similar to pSMAC described in T-B cell-cell conjugates. The functional consequences of ICAM-mediated exploratory contacts on DC activation will be also discussed.

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Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing Th1 and Th2 cell development: dependency on antigen dose and differential Toll-like receptor ligation

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Distinct dendritic cell (DC) subsets have been suggested to be pre-programmed to direct either Th1 or Th2 development, although more recently different pathogen products or stimuli have been shown to render these DC more flexible. It is still unclear how distinct mouse DC subsets cultured from bone marrow precursors or blood, or their lymphoid tissue counterparts direct T helper cell differentiation. We show that mouse myeloid and plasmacytoid pDC cultured from bone marrow precursors, and *ex-vivo* splenic DC subsets can induce the development of both Th1 and Th2 effector cells depending on the dose of antigen. In general, high antigen doses induced Th1 cell development, while low antigen doses induced Th2 cell development. Both cultured and *ex-vivo* splenic plasmacytoid derived DC enhanced $CD4^+$ T cell proliferation and induced strong Th1 cell development when activated with the TLR9-ligand CpG, and not with the TLR4-ligand LPS. The responsiveness of plasmacytoid pDC to CpG correlated with high TLR9 expression, similarly to human plasmacytoid pDC. Conversely, myeloid DC generated with GM-CSF enhanced Th1 cell development when stimulated with LPS, as a result of their high level of TLR4 expression. Thus, the net effect of antigen dose, the state of maturation of the DC together with the stimulation of DC by pathogen-derived products will determine whether a Th1 or Th2 response develops.

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Session 4: Immunotherapy applications of dendritic cells
Chair: Ignacio Melero

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Reciprocal activating interaction between natural killer cells and dendritic cells

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The first response of an organism to infections or other insults to the integrity of the tissues is the activation of the cellular and humoral components of innate resistance including the inflammatory reactions. The cellular interactions and the production of cytokines, chemokines, interferons, and other pro-inflammatory mediators set the stage for the induction of adaptive immunity and largely determine the characteristics of the immune response (e.g. Th1 or Th2, generation of regulatory cells, tolerance). Among the hematopoietic cells acting as effector cells in the innate/inflammatory response, particularly relevant are the phagocytic cells (monocytes, macrophages, neutrophils), the dendritic cells, and the natural killer cells. Dendritic cells have been extensively studied for their exquisite ability to present antigens to T cells, both CD4 and CD8, during the adaptive immune response. However, it is becoming evident that they play an important role as inflammatory cell type in innate resistance, recognizing very early following the infections products of pathogens and secreting an array of pro-inflammatory cytokines. In particular, they trigger the cytokine and cellular cascades that result in the production of either type I or type II interferons. In humans and mouse, in addition to the classic dendritic cells (of which different subsets can be identified), a separate subset of precursor dendritic cells exists, the type I interferon producing cells or plasmacytoid precursor dendritic cells [1,2]. The ability of dendritic cells to respond to pathogens with maturation, activation, and secretion of cytokines is largely mediated by their expression of receptors of the Toll-like family. The different members of this receptor family recognize products from different pathogens. The classic DC and the plasmacytoid DC express distinct pattern of Toll-like receptors suggesting their involvement in their response to different types of pathogens [3]. In particular, the plasmacytoid DC appear to the major cell type responsible for the production of type I interferon in response to several viruses [2,4].

It is of interest that DC not only act as antigen presenting cells for T lymphocytes, but they also interact with the other major effector cell type of innate resistance, the natural killer cells, enhancing their reciprocal activation, maturation, cytotoxic activity, and production of cytokines. In humans, monocyte-derived DC, classic CD11c+ DC, and CD11c- plasmacytoid DC can all be demonstrated to interact with NK cells, participating in their activation, but also being activated by this interaction [5]. The response of the three types of human DC upon interaction with NK cells are distinct, due to their different physiologic characteristics but also due to their pattern of expression of Toll-like receptors and ability to differentially respond to pathogen-derived stimuli.

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Control of T-cell responses by DC-derived Ying and Yang costimulatory signals

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DCs represent a heterogeneous cell population with diverse functions in activation and tolerance of antigen-specific T cell responses. Molecular mechanisms underlying these functions are not yet fully understood. DCs express a rich array of cell surface costimulatory molecules that could activate and inactivate T cell responses. In general, DC-associated costimulatory molecules could be loosely divided into three categories based on their functions. 1) Positive costimulators (CD137L, OX40L, LIGHT, B7h/B7-H2, B7-H3 et al) that augment TCR-mediated T cell growth and differentiation after binding to corresponding receptors. 2) Negative costimulators (B7-H4/B7S1/B7x and others) that inhibit TCR-mediated T cell responses and 3) Dual function costimulators (B7-1, B7-2, B7-H1, B7-DC et al) which could either be stimulatory or inhibitory for a given T cell response. Engaging distinct receptors with opposite signaling functions is believed to be responsible for the operation of dual functions. By differential expression of these ligands on DCs and their receptors on T cells, quality and quantity of T cell responses could be controlled and fine-tuned. Discussion will be focused on new costimulatory pathways and their functions in the context of DC-mediated control of T cell responses.

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Transient and selective aggregation of DRiPs in maturing dendritic cells consequences for MHC Class I presentation

Evelina Gatti and Philippe Pierre

Dendritic cells undergo severe modification of a number of critical functional and biochemical processes in order to maximize their antigen presentation activity in response to a specific stimulation event. It has been demonstrated that DC regulate MHC class II presentation by modulating intracellular traffic according to the maturation state of the cell. On the contrary, little information is available on the ability of the MHC class I presentation pathway to promptly respond to pathogen stimulation other than increase of heavy chain and b2m transcripts. We decided therefore to follow MHCI in DCs at very early time upon stimulation, to identify unknown levels of regulation taking place at the onset of maturation.

MHC class I present peptides derived from ubiquitinated proteins, which are degraded by the proteasome. Of those, the majority is represented by defective ribosomal products (DRiPs), which account for almost 30% of newly synthesized proteins. Recently we have demonstrated the existence of DALIS, specific structures which are formed at the early stages of maturation by the selective aggregation of ubiquitinated proteins. Upon maturation, DALIS rapidly incorporate ubiquitinated DRiPs, which accumulate in the cell although the proteasome degradation rate is not affected. Accumulation of DRiPs is likely to influence MHCI loading. In agreement with this hypothesis, we have shown that at the onset of maturation MHCI-peptide complexes are rapidly and transiently downregulated. The kinetic of this downregulation correlates with the formation of DALIS. At this stage of maturation, the formation of new self-complexes is impaired, as well as preexisting complexes are rapidly lost. At later time, MHC-self peptide complexes start to be formed again, concomitantly with DALIS disappearance.

By segregating transiently self proteins from the proteasome degradation pathway, DALIS might favour MHCI presentation of pathogen-related products, synthesized by the DC or captured by endocytosis and translocated into the cytosol.

This work demonstrates the existence of a new mechanism of regulation of MHC I presentation during DC maturation.

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Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*

Daniel Hawiger, Revati Masilamani and Michel C. Nussenzweig

Dendritic cells (DCs) have the capacity to initiate immune responses, but it has been postulated that they may also be involved in inducing peripheral tolerance. To examine the function of DCs in the steady state we devised an antigen delivery system targeting these specialized antigen presenting cells *in vivo* using a monoclonal antibody to a DC-restricted endocytic receptor, DEC-205. Our experiments show that this route of antigen delivery to DCs is several orders of magnitude more efficient in inducing T cell activation and cell division than free peptide in complete Freund's adjuvant. However, T cells activated by antigen delivered to DCs are not polarized to produce Th1 cytokine IFN-γ and the activation response is not sustained. Co-injection of the DC-targeted antigen and anti-CD40 agonistic antibody changes the outcome from tolerance to prolonged T cell activation and immunity. We conclude that in the absence of additional stimuli DCs induce transient antigen-specific T cell activation followed by T cell deletion and unresponsiveness.

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Cancer immunotherapy using tumor RNA transfected dendritic cells

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Dendritic cells transfected with mRNA encoding tumor antigens stimulate potent CTL responses and tumor immunity in mice. In three phase I/II clinical studies in patients with advanced prostate and renal cancer the majority of patients exhibited specific immunological responses. A number of strategies are currently pursued to enhance the potency and therapeutic impact of this vaccination approach. Two studies will be discussed here: How to induce potent CD4^{+} T cell immunity against endogenously expressed (mRNA-encoded) antigens, and how to reduce the risk for immune escape.

Enhancing CD4^{+} T cell immunity. Recent studies have revealed the pivotal role of CD4^{+} T cells in tumor immunity, yet mRNA transfected DC will favor the generation of CD8^{+} over CD4^{+} T cell responses. We are exploring several strategies to enhance the induction of CD4^{+} T cell immunity using RNA transfected DC. Activation of CD4^{+} T cells corresponding to the mRNA-encoded antigens is achieved by i) re-directing (if necessary) the antigen into the endosomal/lysosomal compartment by appending appropriate translocation signals and ii) transiently inhibiting invariant chain expression in the DC using antisense oligonucleotides or siRNA. In murine studies, these manipulations led to enhanced presentation of class II epitopes, improved CD4^{+} and CD8^{+} T cell induction, and importantly, enhanced antitumor immunity. To enhance the survival and persistence of the CD4^{+} T cells, theantigen-activated CD4^{+} T cells are costimulated via OX40 by co-transfecting the DC with OX40 mRNA ligand. In mice, co-transfection of DC with OX40 ligand mRNA enhances tumor immunity and in human in vitro settings co-transfection of OX40 ligand mRNA enhances the generation CD4^{+} as well as CD8^{+} T cell responses. Current studies are evaluating the combined impact of invariant chain inhibition and OX40L mRNA transfection on the generation and persistence of T cell responses and generation of tumor.

Anti-stromal immunotherapy. Tumor antigens, as currently defined, correspond to gene products expressed in tumor cells. Consequently, an immune response elicited against these antigens will have a direct impact on the tumor. Tumor progression, however, is also critically dependent on the adjacent stroma and therefore immunological targeting of stromal products which are preferentially, though not necessarily exclusively, expressed by the tumor stroma could also impact on tumor growth. Immunological targeting of stromal products, compared to targeting tumor-expressed products, could significantly reduce the incidence of immune escape. We are currently exploring whether stimulating immune responses against angiogenic products can negatively impact on tumor angiogenesis and tumor growth, and

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whether combination immunotherapy targeting angiogenic products and tumor antigens will have a synergistic antitumor impact. Using murine tumor models our studies suggest that immunizing against angiogenic products such as VEGF, VEGFR-2 or Tie-2 inhibits tumor progression in mice without significant adverse effects, and co-immunization targeting angiogenic products and tumor antigens is synergistic.

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Session 5: Dendritic cells in tumor immunotherapy
Chair: Lieping Chen

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Therapeutic manipulation of DC-T cell crosstalk

Drew M. Pardoll

It is now appreciated that dendritic cells are the critical antigen presenting cell involved in the initiation of T cell immune responses by vaccines and other immunotherapeutics. Successful immunization requires efficient presentation by DCs in secondary lymphoid organs. One of the first "DC-based vaccines" to be invented are tumor cell vaccines transduced with the GM-CSF gene. These vaccines secrete GM-CSF at the immunization site, inducing hematopoietic progenitors to differentiate "*in vivo*" into DCs. Clinical development of this approach has been promising and recently, allogeneic GM-CSF vaccines are being actively tested in prostate, pancreas and breast cancer. Analysis of these allogeneic vaccines has demonstrated clearcut evidence of crosspriming in a human vaccine setting. In the case of antigen-specific cancer immunotherapy, tolerance to tumor antigens must be broken and standard vaccine approaches typically fail to transfer antigen to sufficient numbers of DCs. We report a new approach capable of expressing a tumor antigen in every DC in the body. This involves the trasplantation of hematopoietic stem cells transduced with genes encoding tumor antigen. All the DC progeny of transduced HSC express the encoded antigen. After systemic treatment with Flt3 ligand and agonist anti-CD40 antibodies to mobilize and activate DCs *in vivo*, a massive stimulation of antigen-specific T cells is observed. This approach is capable of inducing regressions in established tumors even after tumor specific tolerance is established.

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Intratumoral injection of transfected dendritic cells

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Interleukin-12 (IL-12) is a potent cytokine with many actions that have been exploited in the treatment of experimental rodent malignancies and that have led to clinical trials based on infusion of recombinant heterodimeric protein or in gene-therapy approaches with cassettes of expression encoding for the active molecule. Dendritic cells are producers of IL-12 in a tightly regulated fashion that depends on the maturation status of the dendritic cell with fast on-off kinetics. IL-12 in this setting promotes Th1 differentiation and activation, as well as the generation of activated CTLs and Natural Killer cells. We have established that injections inside malignant tissue of dendritic cells (DC), which have been engineered to produce IL-12 with recombinant adenovirus, induce complete eradication in multiple transplanted established tumors in rodents. This therapeutic activity is dependent on: (i) Antigen capture from malignant cells, (ii) migration to lymph nodes (iii) presence of CTL precursors (iv) the activity of IFNγ. The mechanism of action probably imitates the natural mechanisms of crosspresentation in the priming of T-cells while a grossly exaggerated production of IL-12 takes place at the site of lymphocyte activation. A clinical trial based on those preclinical observations is ongoing at our institution, consisting in repeated intratumor injections of monocyte-derived dendritic cells that have been transfected *in vitro* with a GMP-manufactured adenovirus that encodes for human IL-12. Safety profile, short term clinical follow up and biological effects will be presented. The therapeutic effectiveness can be improved in murine models by repeated dosing, semiallogenic origin of dendritic cells and combination with immunostimulatory anti CD137 monoclonal antibodies.

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Recruiting and loading dendritic cells directly *in vivo* either at the site of vaccination or within the primary tumor

Mario P. Colombo, Cristiana Guiducci, Chiara Massa, Claudia Chiodoni

The ease with which dendritic cells (DC) can be obtained from peripheral blood and loaded with antigens has prompted the start of numerous clinical studies in cancer immunotherapy. This rapid translation to patients has occurred despite several unsolved biological questions regarding the choice of relevant antigen(s), its formulation (cell debris, apoptotic bodies, gene, mRNA, protein, peptide), the way DC are loaded and the maturation stage of DC, their migration *in vivo* and therefore their route of injection. We are trying to solve these critical issues by targeting and loading DC directly *in vivo* either at the site of vaccination or within the primary tumor. Such an approach does not require DC isolation or *in vitro* manipulation and is independent of DC maturation stage since *in vivo*. Moreover, in the case of cellular vaccines, the question of whether apoptotic or necrotic cells are the best immunogens has little relevance *in vivo* since necrosis often follows apoptosis and both conditions are likely to be present simultaneously.

Different strategies are studied to target and load DC directly *in vivo*.

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. This apparently optimal T cell priming is not followed by efficacious therapy. Indeed, C26GM/CD40L cells given for 4 consecutive times after C26 i.v. injection cured 20-40% of mice with lung metastases. 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 and when used irradiated as cellular vaccine cured 85% of mice with lung metastases.

2) Secretion of heath 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, which 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

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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 immunodominat 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 correspondingtumor antigens.

3) Intralesional injection of adenovirus encoding CCL16. Intralesional expression of certain chemokines should attract DC and if concomitant inflammation is induced such DC should be able to mature and migrate to draining lymph node. The liver expressed chemokine (LEC) also known as CCL16 has been inserted into an adenovector and shown to attract CD4, CD8 and DC *in vitro*. Local injection of AdLEC in established (3-4mm, diameter) TSA carcinoma induces pronounced swelling of draining nodes because of the increased cellularity, mainly CD8 and DC producing IFNg and IL-12, respectively. CD8 from the nodes were able of CTL activity against the dominant TAA present on TSA tumor and the DC from the same nodes able to present the same TAA to a specific cell clone. Unfortunately, despite the strong pro-inflammatory activity and the initiation of effective immune response, intralesional administration produces only 30% of complete rejection. While the result might be expected if considering the difficulties of inducing rejection of well established, vascularized tumors, it points to the use of intralesional injection before surgery for prevention of metastases in presence of bad prognostic factors. Experimentally, this can be investigated using the 4T1 spontaneously metastatic mammary carcinoma since surgical removal of small primary tumor is not sufficient to prevent metastatic spread. Injection of AdLEC 5-7 days before surgery results in mice either free or with less than 10 metastases in the lung whereas all mice, in all type of controls, have hundreds of metastases.

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Use of monocyte-derived dendritic cells in cancer immunotherapy

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A novel approach to antigen-specific immunotherapy is to take advantage of dendritic cells (DCs) as "nature's adjuvant" and to actively immunise cancer patients with a sample of their own DC charged with tumour antigens. DC vaccination is, however, still in an early stage, and most potentially important variables (e.g. type and maturational stage of DCs, type of antigen and loading method, schedule etc.) have yet to be addressed. Despite this need for establishing an optimized DC vaccine even in some of the initial exploratory clinical trials, vaccination with DC loaded with tumour antigens in the form of peptides has induced a) tumour-specific killer and helper T cells ("proof of concept"), and b) occasional regression of metastases even in far-advanced cancer patients. This was reported for DC directly isolated from blood as well as for DC generated ex vivo from either CD34+ or CD14+ precursors (reviewed in 1-3). Most of the studies including informative ones in volunteers have been performed by using DC generated from CD14+ monocytes (so-called Monocyte-derived DC or Mo-DC) which are now considered as a gold standard. These Mo-DC can be reproducibly generated within a few days in large numbers (300-800 million mature DC per apheresis) from precursors in blood without any need for pretreating the patients with cytokines like GM-CSF or Flt3-L. Importantly, it is possible to obtain populations of immature Mo-DC by exposing monocytes to GM-CSF + IL-4, which can then be transformed into homogenously mature Mo-DC by various stimuli such as TLR ligands (e.g. microbial products such as LPS or poly I:C), inflammatory cytokines like TNF alpha, monocyte-conditioned medium or its mimic (IL-1beta + TNF alpha + IL-6 + PGE_2), or CD40L. The use of *mature* Mo-DC is likely critical to induce strong immunity as it has become clear that antigen delivered on immature or incompletely matured Mo-DC can even induce tolerance. Interestingly, it has recently also become evident that in case of the Mo-DC the choice of maturation stimulus is probably critical for success. Specifically, PGE_2 has to be part of the maturation stimulus in order to obtain CCR7 expressing Mo-DC that migrate in response to CCL19 and CCL21 that guide DC into lymphoid organs. Using such DC loaded with tumor peptides we have demonstrated their migratory capacity in vivo, the induction of tumor-specific cytotoxic and helper T cells, and the presence of tumor-antigen specific T cells in situ in regressing metastases. Interestingly, immunising to Mage-3A1 peptide by DC has been shown to result in polyclonal T cell responses while other vaccination strategies appear to yielded only monoclonal ones. Currently, we are exploring in two-armed trials whether mature Mo-DC exposed to CD40L and / or an unspecific helper protein (4) influence the quantity or quality of induced T cell responses.

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Recently, new approaches to charge DC with antigens have become evident which promote the DC vaccination approach. The observation that DC can take up naked RNA, express antigens encoded by the RNA and induce antigen-specific T cells in vitro as well as in vivo in patients has given an enormous additional momentum to the use of DC as vectors for antigen delivery and cancer vaccination as this approach now allows one to administer to DC both defined antigens, including universal ones such as telomerase or survivin as well as the total antigenic repertoire of a given tumour (as total tumour or PCR amplified RNA). We have optimised protocols for the electroporation of RNA into both immature and mature Mo-DC, and worked out an intracellular staining method that allows a reliable validation of the resulting DC vaccine. A clinical trial in melanoma patients employing mature Mo-DC transfected with RNA encoding for MelanA, Mage-3 and survivin is ongoing.

The use of dying tumor cells, notably antibody-coated ones, is yet another loading technique. Other preclinical research has shown that the delivery of defined antigens as antigen-antibody complexes to DC enhances crosspresentation and allows for the potent induction of both CD4+ and CD8+ T cell responses. Several recent results suggest that DC might even be useful to directly trigger NK, and to mobilise the additional power of the innate immune system to attack tumour cells. Upon loading with alphaGalactosylCeramide DC can also induce IFN gamma producing NKT cells. It appears, therefore, rational and timely to optimise the use of DC as vectors for the delivery of antigens to vaccinate against cancer.

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Precision guiding of cytolytic T-lymphocyte responses

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

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

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POSTERS

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Phosphatidylinositol 3-kinase regulates the CD4/CD8 T cell differentiation ratio

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The signaling pathways that control T cell differentiation have only begun to be elucidated. Using T cell lines, it has been shown that class IA-phosphatidylinositol 3-kinase (PI3K), a heterodimer composed of a p85 regulatory and a p110 catalytic subunit, is activated following T cell receptor stimulation. Nonetheless, the contribution of p85/p110 PI3K isoforms in T cell development has not been described. Mice deficient in the other family of class I PI3K, p110g, which is regulated by G protein-coupled receptors, exhibit reduced thymus size. Here we examine T cell development in p110g-deficient mice and in mice expressing an activating mutation of the p85 regulatory subunit, p65PI3K, in T cells. We show that p110g-deficient mice have a partial defect in pre-T cell receptor-dependent differentiation, which is restored following expression of the p65PI3K activating mutation. Genetic alteration of both PI3K isoforms also affects positive selection; p110g deletion decreased, and p65PI3K expression augmented, the CD4+/CD8+ differentiation ratio. Finally, data are presented showing that both PI3K isoforms influenced mature thymocyte migration to the periphery. These observations underscore the contribution of PI3K in T cell development, as well as its implication in determining the CD4+/CD8+ T cell differentiation ratio in vivo.

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Activation of immature dendritic cells by Fas-Ligand-bearing gamma/delta T cells

Cheryl Collins, Julie Wolfe, and Ralph Budd

During the differentiation of peripheral blood monocytes to immature dendritic cells (DC) with GM-CSF plus IL-14, the levels of the Fas inhibitor, c-FLIP, increase considerably. This is paralleled by an acquired resistance to Fas-induced death. We have previously observed that c-FLIP can associate with Raf-1 and TRAF-2. As a result, increased expression of c-FLIP can augment activation of the MAP kinase, ERK, and NF-kB. We examined the effects of Fas signaling of immature DC and observe that this stimulates phospho-ERK and production of IL-12 and TNF as well as increased expression of CD80 and MHC class II. This appears to be important during the interaction of gd T cells with DC. gd T cells from inflamed joints in Lyme arthritis express high and sustained levels of Fas-Ligand, and these promote production of the same cytokines from DC.

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Furin, a trans Golgi network protease, mediates TAP independent viral protein processing for antigen presentation to CD8 cytotoxic T lymphocytes

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Recognition of virus-infected cells by CD8+ cytotoxic T lymphocytes (CTL) represents a critical mechanism for virus clearance from the infected host. Viral proteins synthesized within an infected cell are proteolytically processed by proteases. Peptide products are then bound by nascent MHC class I molecules, which transport them to the cell surface for display and recognition by CTL.

The proteasome, whose major role is the degradation of unneeded proteins in the cytosol, is the most abundant and multicatalytic of cellular proteases. As a consequence, it has been considered as the major source of viral peptide epitopes for CTL, even though it has not been evolutionary selected for this task. However, evidence is mounting on the implication of non-cytosolic and non-proteasomal proteases in antigen processing for presentation by MHC class I molecules. Their contribution may expand the repertoire of possible peptides displayed by infected cells to signal them for recognition and elimination by CD8+ T lymphocytes. In addition, it potentially extends epitope processing to other intracellular localizations more accessible to exogenously targeted antigens.

One of the best characterized alternative protease systems is that mediated by the trans-Golgi protease furin (1, 2). Antigen processing of secretory proteins mediated by furin is independent of proteasomes and TAP, and parallels biochemical maturation of antigenic proteins. This vesicular pathway of antigen processing appears to be of general application, since (i) processing by the furin pathway can generate all epitopes tested so far, and (ii) after processing by furin, presentation by a variety of MHC class I molecules to CD8+ CTL takes place. Mechanistical data on this furin-mediated pathway will be presented.

In the context of this Workshop, an intriguing feature of this novel antigen processing pathway is that it operates independently of TAP transporters and separately from the cytosol, in the vesicular compartments. Dendritic cells are critical in the initiation of the CD8+ T-lymphocyte immune response. They do so by what is known as direct priming as well as by cross-priming, by processing and presenting engulfed antigens derived from recently infected cells. As there are antigen endocytic and presentation pathways that are unique to dendritic cells, some of which are TAP-independent, an interesting possibility is that the vesicular,

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furin-mediated pathway of antigen processing contributes to cross-priming by dendritic cells to initiate a full-strength antigen-specific T-cell response.

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VIP affects T helper differentiation acting on dendritic cells

Mario Delgado, Rosa Rodriguez, Doina Ganea

Vasoactive intestinal peptide (VIP), a neuropeptide mainly produced by T helper 2 (Th2) cells following antigenic stimulation, has a general anti-inflammatory effect, both in innate and adaptive immunity. Recent studies indicate that lymphocyte-derived VIP acts as an important T helper-differentiating factor, promoting Th2-type, and inhibiting Th1-type responses. Several non-excluding mechanisms could contribute to the Th2 bias by VIP, acting at the level of Th1/Th2 generation, either directly or through effects on antigen-presenting cells (APCs), and/or at the level of the already generated effectors, by preferentially promoting Th2 proliferation, survival, or accumulation. VIP affects APCs in two ways. First, VIP inhibits production of IL-12 from activated macrophages and dendritic cells (DCs), a cytokine essential for differentiation into Th1 cells. Second, VIP upregulates B7.2 expression in DCs during antigen stimulation, a costimulatory molecule crucial for Th2 differentiation. VIP also promotes the development of Th2 cells by acting directly on differentiating CD4 T cells. In addition, VIP acts on the already generated Th1/Th2 effectors by promoting the survival of Th2, but not Th1 effectors, following antigen stimulation. The VIP-mediated protective effect against antigen-induced apoptosis results in the generation of long-lived memory Th2 cells. Finally, VIP favors directional migration of Th2 through effects on chemokine production by DCs, by inhibiting IP10 and stimulating MDC production. These effects appear to be responsible for the beneficial effect of VIP in models of Th1-type autoimmune diseases such as rheumatoid arthritis and Crohn's disease.

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The human C-type lectin CLECSF8 is a novel endocytic receptor

Ignacio Arce, Laura Martínez-Muñoz, Pedro Roda-Navarro, and Elena Fernández-Ruiz

Cell surface lectin receptors play important roles in the function of macrophages. Herein, we have identified and characterized the human orthologue of the mouse Mcl/Clecsf8. Human CLECSF8 codes for a type II membrane glycoprotein of 215 amino acids that belongs to the human calcium-dependent lectin family (C-type lectin). The cytoplasmic tail of CLECSF8 lacks consensus signaling motifs and its extracellular region shows a single carbohydrate recognition domain (CRD). CLECSF8 gene has been localized on the telomeric region of the NK gene complex on chromosome 12p13 close to MINCLE. CLECSF8 mRNA is slightly expressed in resting Langerhans cells and we are currently studding its regulation. Biochemical analysis of CLECSF8 on transiently transfected cells showed a glycoprotein of 30 KDa. Crosslinking of the receptor leads to a rapid internalization suggesting that CLECSF8 constitutes and endocytic receptor.

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Therapeutic vaccination with autologous monocyte-derived dendritic cells loaded with inactivated autologous virus in patients with chronic HIV infection

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Background: To assess the effects of therapeutic vaccination with autologous MD-DC loaded with autologous HIV on HIV-specific T-cell responses and control of viral replication.

Methods: Autologous MD-DC from patients (median 2x106 cells), obtained from a 60-ml venous blood, were pulsed with autologous heat inactivated HIV-1 (mean 4x106 virions/vaccine), obtained by plasmapheresis 18 months before the vaccination during a period of 3 months of interruption of HAART (STOP 1). Patients (n=12) received 4 subcutaneous doses at 6-week intervals. Thereafter, HAART was discontinued (STOP 2) and patients followed-up during 6 months. Six control patients were also studied.

Results: The vaccination was well tolerated, with one enlargement of local lymph nodes and 2 episodes of flu-like symptoms. There was no a significant decrease of the mean of VL comparing baseline VL (BVL: 4.44 log10) vs set-point VL (4.27 log10), but there was a decrease of set-point VL > 0.5 log in 4 out of 12 patients (0.9, 0.5, 0.7 and 0.8 log) in vaccinated patients. Doubling time of VL rebound increased in vaccinated patients from 1.9 to 2.8 days (p= 0.01) comparing STOP1 vs STOP2. AUC of VL rebound decreased from 3.32 log (STOP 1) to 2.87 log (STOP 2) (p=0.05) in vaccinated patients. No virological changes occurred in controls. There was a significant CD4+ T cell proliferative response to HIV antigens in those patients with a viral load set-point 0.5 log lower than BVL comparing with patients without changes in VL set-point. In contrast, CTL responses decreased significantly after vaccination. After STOP2, LPR and CTL responses were similar to the period before vaccination.

Conclusions: This phase I study showed that: (i) this therapeutic vaccine is feasible, safe and well tolerated; (ii) a partial control of VL was achieved in 1/3 of vaccine recipients and was correlated with a weak but statistically significant increase of CD4 T cell response. Given recent successful data in macaques using an identical vaccination approach a phase II study is required using a much higher amount of HIV to load MD-DC.

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Immunotherapeutic treatment of chronic hepatitis by administration of dendritic cells into chronic WHV carrier woodchucks

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Hepatitis B virus (HBV) infection is a major public health problem and is responsible for about 1.2 million deaths per year worldwide. More than 350 million people throughout the world are chronically infected with HBV and are at high risk of developing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Such individuals could benefit immensely from timely and effective antiviral and immunotherapeutic treatment. Strong antiviral Th cell and CTL responses lead to spontaneous resolution from HBV infection, while patients with chronic infection have weak T cell responses. Thus, the induction of strong immune response could represent an effective treatment modality. Adequate processing and transport of viral antigens by antigen-presenting cells to lymphoid organs is another critical factor for the induction of an efficient antiviral cellular immune response in the host. Professional antigen-presenting cells, especially dendritic cells (DCs), have the unique property to initiate and to modulate this antiviral immune response. The Eastern woodchuck (*Marmota monax*) infected with the woodchuck hepatitis virus (WHV) present a useful model to investigate the basic pathogenesis of acute and chronic HBV infection, including the development of HCC. Chronic WHV carrier woodchucks have been used in the preclinical development of drugs for HBV therapy, in the testing of prophylactic and immunotherapeutic approaches, and in the treatment of HCC. We are studying whether therapeutic vaccination with DCs transduced to express viral antigens Using a mouse model we have shown that systemic administration of DCs infected with a recombinant adenovirus construct encoding for Hepatitis virus Core antigen induces a cellular Th2 type immune response and only when the DCs are co-transduced with an adenovirus expressing the cytokine IL-12 this antigen specific cellular immune response is biased to a type 1 protective immune response. Meanwhile we have developed a protocol to obtain DCs from woodchuck peripheral blood which in the absence of surface markers have been characterized by its function and morphology.

Conclusions: Adoptive transfer of WHV core antigen pulsed DCs induced a protective cellular immune response only when an immunostimulatory cytokine such us IL-12 is co-administered.

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Plasmacytoid dendritic cells are more closely related to B cells than classical dendritic cells with respect to their molecular regulation of CIITA and MHCII expression

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Major histocompatibility complex class II (MHCII) molecules play a pivotal role in the induction and regulation of immune responses. All qualitative and quantitative aspects of MHCII expression are controlled by the class II transactivator (CIITA). The gene encoding CIITA (MHC2TA) is controlled by three distinct and independent promoters (pI, pIII, pIV), which are transcribed in a tissue-specific manner. IFN-g-induced CIITA expression is mediated by pIV. pIII is used mainly in B cells. Expression of the MHC2TA gene in dendritic cells (DCs) is controlled primarily by pI.

In order to study the specificity and functional importance of pI in vivo we have generated a new strain of knockout mice in which CIITA promoters pIII and pIV haven been excised (pIII+IV -/- mice). Only pI-driven CIITA expression is retained in these mice. As a consequence of the loss of pIV, IFN-g-induced CIITA and MHCII expression is completely abolished in all non-bone marrow-derived cells. In addition, the mice lack constitutive MHCII expression on the cortical epithelial cells of the thymus and thus lack positive thymic selection of T cells. Furthermore, as a consequence of the loss of pIII, all B cells in the pIII+IV -/- mice are CIITA and MHCII negative. However, DCs isolated from the spleen and thymus or generated in vitro from bone marrow of pIII+IV -/- mice express CIITA and MHCII at normal levels. They present antigens and activate T cells with the same efficiency as DCs from control mice. Surprisingly, CD11c+ B220+ GR1+ plasmacytoid DCs (pDCs) isolated from the spleen, thymus or bone marrow of pIII+IV -/- mice are completely devoid of CIITA and MHCII expression. As it is the case for B cells, expression of the MHC2TA gene in pDCs is controlled by pIII rather than by pI. pDCs can thus be uncoupled from classical CD11c+ DCs in terms of their molecular regulation of CIITA and MHCII expression. These new findings will allow us to address as yet unresolved questions concerning the role of antigen presentation and activation of CD4+ T cells by pDCs in vivo.

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Origin and differentiation of dendritic cells

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Dendritic cells (DCs) are key components of the immune system owing to their essential role in the induction and control of T cell immunity, as well as in the modulation of B cell and NK cell responses. Research on DCs over the last decade has led to the concept that the DC system comprises a large collection of subpopulations with different functions. Functional specialization allows defined DC subsets to induce efficient defence mechanisms against pathogens and tumor cells and maintain T cell tolerance. A key question, with important implications for our understanding of the induction and control of immunity by DCs, as well as the use of DCs for immunotherapy, is whether DC functional diversity results from the existence of developmentally independent DC subpopulations, or whether DC subsets sharing a common differentiation origin acquire specific functions in response to environmental signals. Although a definitive model of DC development remains to be established, recent data derived mostly from *in vivo* DC reconstitution assays in the mouse indicate that the same DC subpopulations, including CD8- DCs, CD8+ DCs and B220+ plasmacytoid DCs, can be derived from either myeloid or lymphoid progenitors. Therefore these experiments do not support the existence of independent myeloid and lymphoid DC subpopulations as previously proposed, but a DC differentiation model relying on a dual contribution of myeloid and lymphoid differentiation pathways.

Interestingly, recent data from our laboratory suggest that during microbial infections, blood-borne DC precursors are recruited to the reactive sites where they differentiate into fully competent DCs.

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Localization of the neuronal protein Kidins220 in a raft compartment at the leading edge of motile immature dendritic cells

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The Kinase D-interacting substrate of 220 kilodaltons (Kidins220) is the first physiological substrate for protein kinase D (1). Kidins220 is an integral membrane protein that contains four transmembrane domains and cytoplasmic regions that bear ankyrin repeats, a proline rich region and putative phosphorylation sites for serine-threonine kinases (1). Kidins220 is predominantly expressed in neural tissues, and in differentiated PC12 cells it is found at the tip of the extending neurites. We show that Kidins220 is expressed in monocyte-derived and in peripheral blood dendritic cells (DCs). Immature DCs (iDCs) migrate onto extracellular matrices changing cyclically along the process from a polarized morphology (monopolar stage) to a morphologically symmetrical shape (bipolar stage). Kidins220 localized on membrane protrusions at the leading edge or on both poles in monopolar and bipolar iDCs, respectively. F-actin co-localized and it was necessary for Kidins220 localization on the membrane edges of monopolar and bipolar cells. In monopolar cells, rafts were observed at the leading edge. In bipolar cells, rafts located either in a perinuclear region or on one pole. Membrane Kidins220 and rafts co-localized only when the latter were found on one pole in monopolar or bipolar cells, suggesting that rafts may be markers and/or determinants of the leading edge. Disruption of rafts by treatment with methyl-b-cyclodextrin induced rounding of the cells, inhibition of motility and lost of Kidins220 polarization. The localization of Kidins220 at the leading edge suggests that this molecule may be involved in regulating motile-related functions in DCs. As Kidins220 seems to have a similar localization in PC12 cells, we speculate that this surface protein might regulate similar motile function/s in DCs and neuronal cell.

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Migration of human blood dendritic cells across endothelial cell monolayers

de la Rosa, G., Longo, N., and P. Sánchez-Mateos

Distinct subsets of dendritic cells (DCs) are present in blood, probably "en route" to different tissues. We have investigated the chemokines and adhesion molecules involved in the migration of myeloid (CD11c+) and plasmacytoid (CD123+) human peripheral blood DCs across vascular endothelium. Among blood DCs, the CD11c+ subset vigorously migrated across endothelium in the absence of any chemotactic stimuli, whereas spontaneous migration of CD123+ DCs was limited. In bare cell migration assays, myeloid DCs responded with great potency to several inflammatory and homeostatic chemokines, whereas plasmacytoid DCs responded poorly to all chemokines tested. In contrast, the presence of endothelium greatly favored transmigration of plasmacytoid DCs in response to CXCL12 (SDF-1) and CCL5 (RANTES). Myeloid DCs exhibited a very potent transendothelial migration in response to CXCL12, CCL5, and CCL2 (MCP-1). Furthermore, we explored whether blood DCs acutely switch their pattern of migration to the lymph node-derived chemokine CCL21 (SLC) in response to microbial stimuli (viral dsRNA or bacterial CpG-DNA). A synthetic dsRNA rapidly enhanced the response of CD11c+ DCs to CCL21, whereas a longer stimulation with CpG-DNA was needed to trigger CD123+ DCs responsive to CCL21. Use of blocking monoclonal antibodies to adhesion molecules revealed that both DC subsets used PECAM-1 to move across activated endothelium. CD123+ DCs required b2- and b1-integrins to transmigrate, whereas CD11c+ DCs may use integrin-independent mechanisms to migrate across activated endothelium.

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Improving efficacy of Interleukin-12-transfected dendritic cells injected into murine colon cancer with anti-CD137 and alloantigens

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Aim of study:

Intratumoral injection of dendritic cells (DC) genetically modified to produce Interleukin-12 (IL-12) has been shown to elicit an immune response which eradicates established subcutaneous tumors in several murine models. Here we searched for different strategies that could achieve better antitumor efficacy.

Materials and Methods:

Therapies were tested in subcutaneous unilateral or bilateral established tumors derived from CT26 and MC38 murine colon carcinomas. DCs were cultured from bone marrow, transfected to produce IL-12 with recombinant adenovirus and injected intratumorally. Migration of DCs was traced using DCs from EGFP-transgenic mice. Tumor sizes were followed and the number of IFN-gamma-secreting lymphocytes responding to tumor cells was determined by ELISPOT.

Results:

Repeated injections of IL-12-secreting DC as opposed to a single injection achieve better efficacy both against the injected and a distantly implanted tumor. The use of semiallogeneic DC, that are mismatched in one MHC haplotype with the tumor host, showed slightly better efficacy than autologous DC in the same therapeutic strategy. The combination of intratumoral IL-12-producing DC with systemic injections of immunostimulatory anti-CD137 (4-1BB) monoclonal antibody achieves potent synergistic effects that correlate with the increased number of IFN-gamma-secreting lymphocytes. The elicited systemic immune response eradicates concomitant untreated lesions in most cases. Repeated doses of IL-12-secreting semiallogeneic DC plus anti-CD137 monoclonal antibody were used to treat bilateral tumors established for two weeks, showing bilateral curative efficacy in some cases (2 out of 6 cases).

Conclusion:

Repeated doses, semiallogeneic origin and combination with anti-CD137 monoclonal antibody enhance antitumor therapeutic efficacy of intralesionally delivered DCs engineered to produce IL-12. These approaches increase the efficacy of a therapeutic strategy that is currently being tested in clinical trials.

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Maturity and antigen presenting properties of lymphoid organ dendritic cells

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A systematic assessment of the maturity and the antigen presenting properties of lymphoid organ dendritic cells (DC) has been lacking. We have analyzed the maturity of the DC types found in the steady state in the spleen, lymph nodes (LN) and thymus. The DC that migrate into the iliac, mesenteric, mediastinal or subcutaneous LN from peripheral tissues were mature, so they could not process and present newly encountered antigens. However all the other DC types (which accounted for most of the splenic and thymic DC, and approximately half of the LN DC) were phenotypically and functionally immature: they expressed low levels of surface MHC II and CD86, accumulated MHC II in their endosomes, and could present newly encountered antigens. These immature DC could be induced to mature by culture *in vitro* or by inoculation of inflammatory stimuli *in vivo*. Therefore the lymphoid organs contain a large cohort of immature DC, most likely for the maintenance of peripheral tolerance, which can respond to infections reaching those organs and mature in situ (1).

We have also analyzed the mechanisms that control the developmental regulation of antigen presentation in conventional and plasmacytoid DC *in vivo*. Conventional immature DC constitutively presented self antigens, but their MHC II-peptide complexes were quickly degraded after their transient expression on the cell surface. Upon activation, endocytosis of the MHC II-peptide complexes was selectively downregulated and MHC II synthesis was gradually turned off. This resulted in the accumulation of newly generated MHC II-peptide complexes on the surface of the mature DC, where they were long-lived. Mature conventional DC can thus display for a long time a "snapshot" of the antigenic material they captured at the site and time of activation (2,3). In contrast, the plasmacytoid DC up-regulated MHC II synthesis upon activation, and their MHC II-peptide complexes were continually turned-over. This biased the antigen presenting activity of plasmacytoid DC to endogenously synthesized proteins.

Finally, we have determined the capacity of the lymphoid organ DC to cross-present (i.e. present exogenous antigens via MHC I molecules). Only the CD8+ DC from the spleen, LN and thymus could cross-present with high efficiency independently of whether the antigen was cell-associated or soluble. Therefore, CD8+ DC are uniquely and constitutively equipped to direct exogenous antigens to the MHC I presentation pathway independently of the form of antigen (4).

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The lectures summarized in this publication were presented by their authors at a workshop held on the 6th through the 8th of October, 2003, at the Instituto Juan March.

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