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Karolinska Institutet, Stockholm, Sweden

PROCESSING AND PRESENTATION OF EXOGENOUS ANTIGEN BY DENDRITIC CELLS

LIYING CHEN M.D.

Karolinska Institutet

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To my family

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Chen L**, Jondal M. Endolysosomal processing of exogenous antigen into major histocompatibility complex class I-binding peptides. *Scand J Immunol*. 2004; 59:545-52.

II. **Chen L**, Jondal M. Alternative processing for MHC class I presentation by immature and CpG-activated dendritic cells. *Eur J Immunol*. 2004; 34:952-60.

III. **Liying Chen** and Mikael Jondal. Vesicular MHC class I cross-presentation by activated dendritic cells. (Manuscript)

IV. **Liying Chen**, Elisebeth Darré, Betil B Fredholm and Mikael Jondal. Adenosine, through the A_1 receptor, inhibits cross-priming by dendritic cells by the retention of intracellular peptide/MHC-1 complexes. (Manuscript)

V. Wallner FK*, **Chen L***, Moliner A, Jondal M, Elofsson M. Loading of the antigen-presenting protein CD1d with synthetic glycolipids. *Chembiochem*. 2004; 5:437-44.
***** These authors contributed equally.

Related publication

Chen L, Sundback J, Olofsson S, Jondal M. Interference with O-glycosylation in RMA lymphoma cells leads to a reduced in vivo growth of the tumor. *Int J Cancer*. 2006 Apr 13; [Epub ahead of print]

Abstract

Dendritic cells (DC) play a key role in the immune system. In this thesis, we have investigated and characterized pathways for the processing and presentation of exogenous OVA on MHC-I ("cross-presentation") and how these were influenced by the purine nucleoside Adenosine (Ado), an immune regulator that accumulates to high concentrations at sites of inflammation and tumor growth. In addition, we established a method to detect the binding of glycolipids containing tumor-associated carbohydrate antigens (TACAs) to conserved CD1d molecules expressed on viable mouse cells, including DC.

Immature (iDC) and activated (aDC), but to a lesser degree resting DC (rDC), were found to express a fully functional, highly efficient TAP-independent vesicular MHC-I processing pathway (VP). In this VP, OVA-derived SIINFEKL/Kb complexes were formed through peptide exchange in recycling Kb molecules. Such complexes were also formed at the cell surface by peptide regurgitation. This high efficient VP could be restored in rDC by cellular activation using TLR ligands, such as CpG (binding to TLR-9) and LPS (binding to TLR-4) and also by the mere mechanical disruption of cells, the latter occurring without the development of an activated phenotype and the secretion of IL-12 in cells. Reactivation of the high activity VP in rDC was not dependent on type I interferon or IL-12 but required the adaptor protein MyD88. We also confirmed that the cysteine protease Cathepsin S (Cat S) was required for the formation of the SIINFEKL/Kb complexes in the VP. In addition, we found that Ado had a strong inhibitory effect on the low degree VP seen in rDC and that this effect was mediated by the A_1 Ado receptor and involved the retention of intracellular SIINFEKL/Kb complexes.

Four newly synthesized TACA-containing glycolipids were studied for the binding to CD1d on viable cells by the use of specific anti-TACA mAbs and flow cytometry. By this procedure specific binding could be visualized and quantitated. A method like this might be useful for the controlled loading of glycolipid antigens into DC and the possible use of such cells for immunotherapy.

Key words: DC, vesicular MHC-I processing, Adenosine, CD1d, TACA

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ado	Adenosine
APC	Antigen presenting cells
pAPC	Professional antigen presenting cells
Cat S	Cathepsin S
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
iDC	Immature dendritic cells
rDC	Resting dendritic cells
aDC	Activated dendritic cells
ER	Endoplasmic reticulum
αGalCer	α-galactosylceramide
GL	Glycolipid
GM-CSF	Granulocyte-monocyte colony-stimulating factor
IFN	Interferon
IL-4	Interleukin-4
LC	Langerhans cells
LPS	Lipopolysaccharide
LN	Lymph node
MHC-I	Major histocompatibility complex (MHC) class I
β_2-m	β_2-microglobulin
NKT	Natural killer T cells
OVA	Ovalbumin
PRR	Pattern recognition receptor
TACA	Tumor-associated carbohydrate antigen
TAP	Transporter-associated with antigen processing
TLR	Toll-like receptor
VP	Vesicular MHC-I processing pathway

INTRODUCTION

1. The immune system

1.1 Definition

Immune means resistance to a particular infectious disease as a consequence of an earlier infection with the same microbe or after a vaccination process using the corresponding antigen. The physiologic functions of the immune system are thus to prevent and eradicate infections. However, the impact of the immune system goes beyond infectious disease, in that it involves also non-infectious situations for instance, transplantation, autoimmunity, hypersensitivity diseases and tumor immunotherapy (1).

1.2 Anatomy of lymphoid organs and tissues

The lymphoid organs and tissues of the immune system are stationed throughout the body as illustrated in Figure 1.

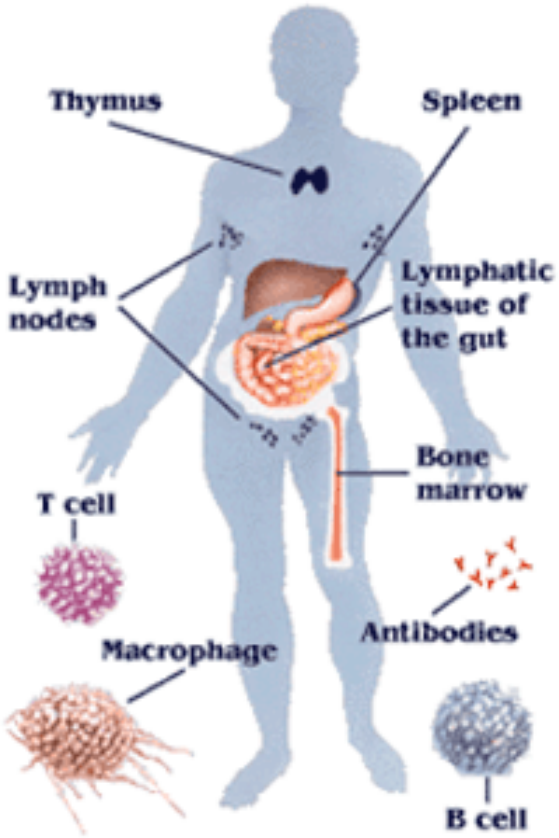

Figure 1 Anatomy of lymphoid organs and tissues.

They include thymus, bone marrow, lymph nodes (LN), spleen, mucosal-associated lymphoid tissues (MALT) and cutaneous immune system. Lymphoid organs or tissues are classified as generative (primary or central) such as thymus and bone marrow, where T and B lymphocytes mature, respectively and peripheral (secondary) such as LN, spleen and MALT where adaptive immune responses are generated. The blood and lymphatic vessels that carry lymphocytes can also be considered as part of the lymphoid system.

1.3 The innate immune system

1.3.1 Characteristics

Host defense mechanisms consist of innate immunity, mediating the initial protection and adaptive immunity, conducting a later and more specific immune responses. Innate immunity, also called natural or native immunity, is always present in healthy individuals, prepared to block the entry and rapidly eliminate microbes. It not only provides the early defense against infections but also instructs the adaptive immune system to effectively combat infections. The components of the innate immune system include epithelial barriers, natural antibiotics, circulating or tissue-resident phagocytes (neutrophils and monocytes/macrophages), NK cells, plasma proteins, including the proteins of the complement system, innate cytokines and others.

1.3.2 Recognition of microbes by the innate immune system

Receptors of the innate immune system recognize structures that are shared by various microbes, but not present on host cells. These structures are often essential for the survival and infectivity of these microbes. The receptors of the innate immune system are encoded in the germline and have a limited diversity in contrast to the high diversity of antigen specific receptors displayed on B and T cells generated through somatic gene rearrangements. The germline-encoded microbial pattern recognition receptors (PRRs) have evolved as a protective adaptation to potentially harmful microbes in the environment. Some innate receptors are expressed by phagocytes for stimulating the phagocytosis and killing of the microbes. The mannose receptor is a macrophage lectin that binds terminal mannose and fucose residues of glycoproteins or glycolipids expressed on microbial cell walls. The macrophage scavenger receptor binds a variety of

microbes. Antibodies and some complement components can opsonize microbes and thus promote the phagocytosis of these.

1.3.3 Toll-like receptors (TLRs)

Being part of the PRRs, TLRs recognize specific pathogen-associated molecular patterns (PAMPs) and also activate phagocytes including dendritic cells (DC) after recognition. To date, 10 in humans and 13 in mice TLRs are identified. TLR1/TLR2 and TLR2/TLR6 recognize various bacterial components of Gram-positive bacteria and mycoplasma lipopeptide. TLR3 recognizes double-stranded RNA (dsRNA) produced during virus replication. TLR4 recognizes LPS of Gram-negative bacterial. TLR5 recognizes bacterial flagellin. TLR7/8 recognize single-stranded RNA (ssRNA). TLR9 recognizes bacterial and viral CpG DNA motifs (2-4). After recognition of microbial pathogens, TLRs trigger intracellular signalling pathways that result in the inductions of inflammatory cytokines, type I interferon and chemokines as shown in Figure 2, as well as up-regulation of co-stimulatory molecules.

TLRs contain extracellular leucine-rich repeats responsible for the recognition of pathogens, the trans-membrane and the cytoplasmic Toll/interleukin-1 receptor (TIR) domains required for initiating intracellular signalling. TLR-mediated intracellular signalling is initiated by the TIR-domain-dependent interaction with adaptor proteins MyD88 (myeloid differentiation factor 88), TIRAP/Mal (TIR domain-containing adaptor protein), Trif (TIR domain-containing adaptor inducing IFNβ) and TRAM (Trif–related adaptor molecules). MyD88 is a central adapter protein shared by almost all TLRs. TIRAP/MAL is required for the activation of MyD88-dependent pathway, while TRAM is required for the Trif-dependent pathway. MyD88 recruits IRAK (interleukin-1 receptor-associated kinase) proteins and TRAF6 (tumor necrosis factor receptor-associated factor 6) upon ligand stimulation and further leads to NF-kβ activation (MyD88-dependent pathway). TLR4 also recruits TRAM and Trif, which interacts with IRF3 (interferon regulatory factor 3). Phosphorylated IRF3 is then translocated into nucleus (Trif-dependent pathway). Manipulation of TLR system may contribute to the development of vaccine therapy (2, 5-8).

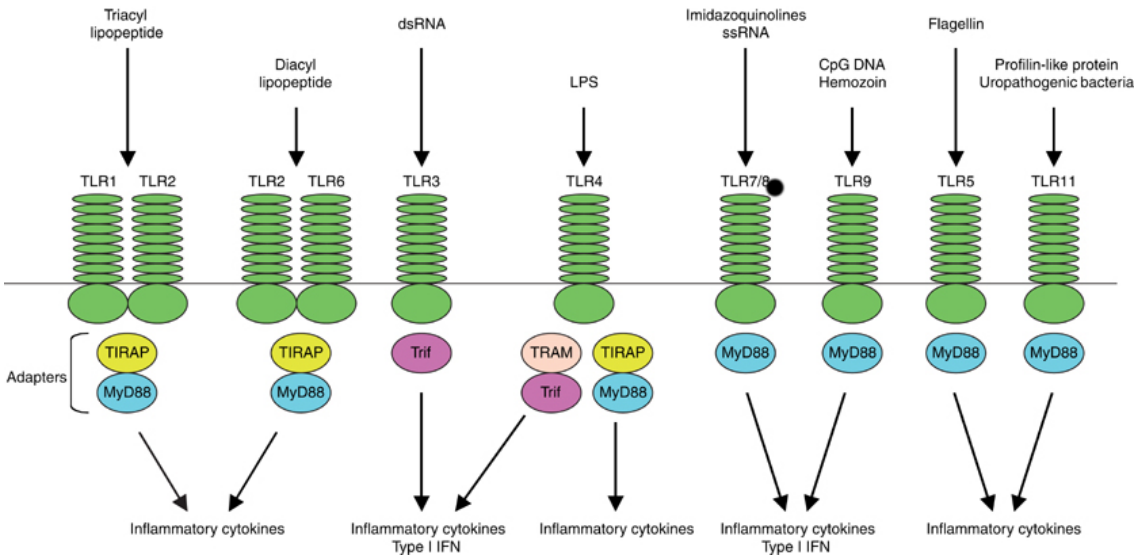

Figure 2 TLR-mediated immune responses (Adapted from Akira 2006)

1.4 The adaptive immune response

1.4.1 Humoral and cellular immunity

Adaptive immunity, also called specific or acquired immunity is generated in response to microbes that invade tissues. Properties of the adaptive immunity are the expression of extremely diverse lymphocyte repertoires and the generation of memory. There are two types of adaptive immunity, called humoral immunity and cell-mediated immunity, designed to provide defense against extracellular and intracellular microbes, respectively.

Humoral immune response is mediated by antibodies, secreted into the circulation and mucosal fluids by activated B lymphocytes. Antibodies function by neutralizing microbes and microbial toxins, as well as activating the complement system. B cells are able to respond to basically any form of antigens, including proteins, carbohydrates and lipids through their surface antigen receptors (BCR), whereas most T cells can only recognize processed protein antigens by their T cell antigen receptors (TCR).

Cell-mediated immune responses are accomplished by T lymphocytes which can be subdivided into $CD4^+$ and $CD8^+$ T cells according to their co-receptor expression on the cell surface. $CD8^+$ T cells can be further differentiated into cytotoxic T lymphocytes (CTL) to kill the target cells, whereas $CD4^+$ T cells can be further developed into either subsets of helper cells, including Th1, Th2 or even regulatory T cells (9. 10). IFN-γ, produced by Th1 cells can activate macrophages to increase killing of phagocytosed microbes. Th2 cells can drive B cells towards antibody production, isotype-switching as well as affinity maturation. Regulatory T cells function to suppress excessive immune response and prevent autoimmunity.

1.4.2 Antigen presenting cells (APC)

The adaptive immune responses are initiated by the recognition of antigenic peptides displayed by MHC molecules on antigen presenting cells (APC). Basically, any cell types that express MHC can process antigens for the display of the complexes on the cell surface, but not all of them can initiate the effector functions of B and T cells in adaptive immune response. Professional antigen presenting cells (pAPC), including DC, macrophages and B cells are not only capable of processing antigens, but also capable of priming immune effector cells to different extents.

There are two major cellular pathways for the processing and presentation of protein antigens on MHC-I or MHC-II molecules (see further in section 3). The classical MHC-I pathway present endogenous, cytosolic antigens, whereas the MHC-II pathway process exogenous antigens. However, MHC-I restricted T cell responses can also occur in response to exogenous antigens, in a process called “cross-priming”, by the use of alternative processing pathways. A major focus in the present thesis has been to study such alternative pathways in mouse DC.

2. Dendritic Cells (DC)

2.1 DC history

The name of dendritic cells originates from the Greek word *dendron*, meaning tree and appropriately describes the distinct morphology with many extensions. This cell type was first visualized in the skin in 1868 by Paul Langerhans (11) and was named Langerhans cell (LC) after his name. It was rediscovered by Steinman and colleagues (12) in 1973 in the course of observations on the cells of mouse spleen, realizing that *these novel cells can assume a variety of branching forms, constantly extend and retract many fine cell processes*. The term dendritic cells (DC) for this particular cell type was suggested.

2.2 DC origins and subsets (mouse DC)

DC derive from bone marrow hematopoietic progenitors. The DC system comprises a large collection of subpopulations with different functions. Functional diversity of DC population is related to their differentiation stage, as well as their specific location. However, question still remains whether the functional diversity of DC results from developmentally independent subpopulations (lineage model) or a common differentiation origin but acquiring specific functions in response to environmental signals (plasticity model) or a mixture of these two extreme models (13-15).

A large variety of DC subsets have been described in lymphoid and non-lymphoid organs. Some subsets seem specific to defined tissue environments. Both phenotypic and functional criteria have allowed the classification of mouse DC into at least six main subpopulations in lymphoid organs, as described in Table 1, including the mature forms of the peripheral nonlymphoid tissue-resident DC. Three types of DC are consistently found in the spleen: CD8 DC, CD4 DC and double negative DC (DN DC) and two extra DC subtypes, not normally found in the spleen are in the lymph nodes (LN), which have apparently arrived in the LN through the lymphatic vessels. They are $CD4^{+}CD8^{-}$ $CD205^{moderate}$ $CD11b^{+}$ DC and $CD4^{-}CD8^{low}CD205^{hi}Langerin^{+}CD11b^{+}$ DC, the mature forms of the tissue interstitial DC (including dermal DC) and Langerhans DC (LC), respectively (14-16) (Table1).

Table 1 Major lymphoid organ distribution of mouse DC subpopulations

	Spleen	Lymph node	Thymus	Blood
$CD4^-CD8^+CD205^{hi}CD11b^-$ DC (CD8 DC)	+	+	+	-
$CD4^+CD8^-CD205^-CD11b^+$ DC (CD4 DC)	+	+	-	-
$CD4^-CD8^-CD205^-CD11b^+$ DC (DN DC)	+	+	-	-
$CD4^+CD8^-$ $CD205^{moderate}$ $CD11b^+$ DC (Mature form of interstitial/dermal DC)	-	+	-	-
$CD4^-CD8^{low}CD205^{hi}Langerin^+CD11b^+$ DC (Mature form of Langerhans DC)	-	+	-	-
Plasmacytoid B220+ DC (pDC)	+	+	+	+

CD8 DC accumulates in the T cell area of a given peripheral lymphoid tissue, while CD4 and DN DC are located in the marginal zones. CD8 DC constitutively capture antigens from DC that migrate in the steady-state into the T cell area, whereas marginal DC may migrate to the T cell area upon stimulation with microbial products, which is also seen with CD8 DC in the T cell area. These three types of DC, found in the spleen and lymph nodes may derive from blood DC precursors that seed the spleen and LN directly from the bone marrow via blood. As spleen is only accessible via blood, these DC do not seem to immigrate from peripheral tissues before reaching the spleen. These cells are completely replaced within 2-5 days requiring a considerable input of DC precursors to maintain the homeostasis of DC in the spleen (16).

Langerhans Cells (LC), derived from DC precursors from the blood, pick up, process the antigens captured in the periphery and bring this information to the draining lymph nodes for the communication with naïve T cells. In the steady-state DC present self-antigens for the regulation of T cell homeostasis and T cell tolerance (17-21).

Thymic DC The CD8 DC is the dominant subtype in the thymus. Thymic DC were proposed to derive from an intrathymic precursor, develop and die within the thymus. Their function is to induce central tolerance of T cells by inducing the apoptotic death of potentially self-reactive T cells (14).

pDC Plasmacytoid $B220^+$ DC (pDC) precursors are found in all lymphoid organs and blood. pDC are able to produce large amount of type I interferon (IFNα/β) when stimulated with bacterial CpG or by viral infection. pDC can be distinguished from the other DC by the expression of CD45RA and CD11c at intermediate levels. Although they resemble the normal CD8 DC in many respects, arguments still remain whether pDC are the precursors of CD8 DC in normal uninfected stage. The longer half-life (around 9 days) and the requirement of an exogenous stimulus make it difficult to study this DC progeny in uninfected laboratory mice (14, 22).

Human DC In contrast to mouse DC, human DC were mostly isolated from blood. Blood is a source of immature DC (iDC) and pDC. Human blood DC are heterogeneous in the expression of a range of markers, but many of these reflect differences in maturation or activation stages, rather than separate sublineages. Human DC lack the expression of CD8, so the counterpart of mouse CD8 DC remains elusive. In addition, human Langerhans cells are recognized as a separate DC subtype with distinct makers, including CD1a and langerin. In a few cases, DC were isolated from spleen and tonsillar, showing the herogeneity in the expression of CD4, CD11b and CD11c, indicating a complexity resembling mouse splenic DC. Most human thymic DC are $CD11^+CD11b^-CD45RO$ and lack of myeloid markers, resembling the mouse thymic CD8 DC. A minority DC in the thymus, $CD11c^{high}CD11b^+CD45RO^{high}$ express myeloid markers, resembling mouse $CD8^-$ myeloid DC (14).

2.3 DC generation and differentiation in vitro (mouse DC)

DC constitute less than 0.1% of blood leukocytes (23) and are sparsely distributed all over the body. Moreover, they do not proliferate in peripheral tissues. Accordingly,

methods developed to generate bulk DC in vitro from precursors in the bone marrow, spleen or blood represent a major breakthrough in studies of DC biology.

DC develop from hematopoietic precursor cells through successive steps of lineage commitment and differentiation. Different experimental approaches have been designed to study the development of murine DC in vitro, ranging from different hematopoietic precursors, diverse DC differentiation/maturation-inducing mediators to different culture protocols. Most methods developed for generating DC in vitro rely on two-step culture protocols, generating immature DC after first culture period involving cell proliferation, followed by a second incubation phase leading to mature DC, in which no expansion takes place (24). Main protocols (mouse DC) are shown in Figure 3.

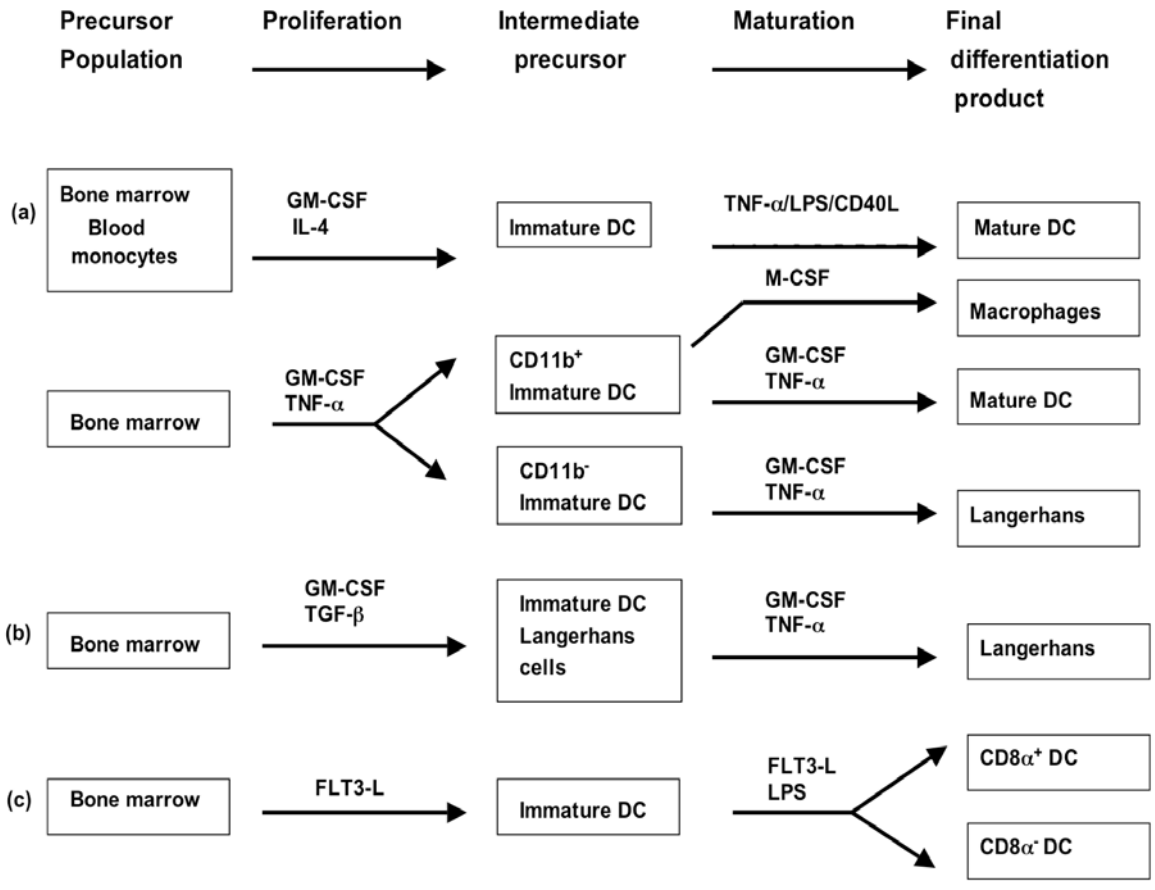

Figure 3 Main in vitro differentiation pathway for mouse DC

The first protocol for the generation of bulk DC from mouse bone marrow was described by Inaba K et al in 1992 (25), using recombinant GM-CSF and IL-4 and further maturation of cells using tumor necrosis factor α (TNF-α). The generated DC population is phenotypically homogeneous and does not express CD8α. Maturation can also be induced by lipopolysaccharide (LPS) and CD40L. This method leads to the differentiation of DC with phenotypic and functional characteristics similar to those described for human monocyte-derived DC. Alternatively, DC can be produced using GM-CSF, together with TNF-α and two DC subsets with different phenotypes are generated as described in Figure 3. LC-like DCs can be differentiated in the presence of GM-CSF and TGF-β and further stimulated with TNF-α. The FLt3 ligand has been shown to generate pDC-like DC after maturation with either LPS or IFN-α. Both $CD8\alpha^-$ and $CD8\alpha^+$ DC are generated by this method (24).

Cytokines in DC generation and differentiation in vitro

GM-CSF Being one of the first cytokines identified to have effect on DC, GM-CSF is a central mediator for the generation of DC in vitro. GM-CSF is a 20-30 kDa glycoprotein synthesized by lymphocytes, monocytes, fibroblasts and endothelial cells. Its major function appears to prolong cellular survival. It promotes the differentiation of monocytes to large macrophage-like cells, increasing their metabolism and function as APC by enhancing MHC-II. GM-CSF is mainly used in generation of DC from bone marrow and blood in vitro (26, 27).

IL-4 IL-4 is a 18-20-kDa glycoprotein that is produced primarily by activated T cells. It is the principal factor controlling the differentiation of monocytes into DC, as monocytes cultured in the presence of IL4 acquire a macrophage–like DC morphology. Upon exposure to IL-4, cells increase in size and develop extensive processes. In general, IL-4 suppresses overgrowth by macrophages, allowing for the generation of human DC from peripheral blood. Specifically, IL-4 inhibits macrophage colony formation, whereas GM-CSF alone induces the formation and survival of aggregates of DC progenitors (26, 27).

TNFα TNFα is a 17-kDa protein produced primarily by macrophages. It is mainly used in culturing $CD34^+$ cells. Culturing $CD34^+$ cells in the presence of TNFα results in adherent and non-adherent cells with DC features, whereas without, these cells differentiate to monocytes, macrophages and granulocytes. TNFα delivers critical signal to DC differentiation from monocytes (26, 27).

Flt3-L FL is a member of a small family of growth factors, stimulating proliferation of hematopoietic cells and mobilizing stem and progenitor cells to peripheral blood. Flt3-L increases the absolute numbers of mature myeloid precursor-derived DC generated from $CD34^+$ bone marrow progenitors (26, 27).

TGF-β TGF-β is a suppressive cytokine totally blocking DC maturation from mouse bone marrow. DC generated in the presence of TGF-β are immature, but TGF-β did not inhibit the commitment of progenitor cells to the DC lineage (26, 27).

Other cytokines have also been used in generation of DC in vitro with different characteristics, including IL-3, IL-1, IL-10, IL-7, IL-15 and CD40 ligand (26, 27).

2.4 DC migration

DC, in order to accomplish their multi-biologic functions need to undergo a complex pattern of migration. DC migration includes three steps: migration of circulating DC precursors which extra-vasate from the blood to the lymphoid and non-lymphoid tissues or organs, migration from local sites of inflammation to secondary lymphoid organs and lastly the positioning of DC for interacting with naïve and memory T cells in the T cell areas in lymphoid tissues or organs, as some of the steps shown in Figure 4.

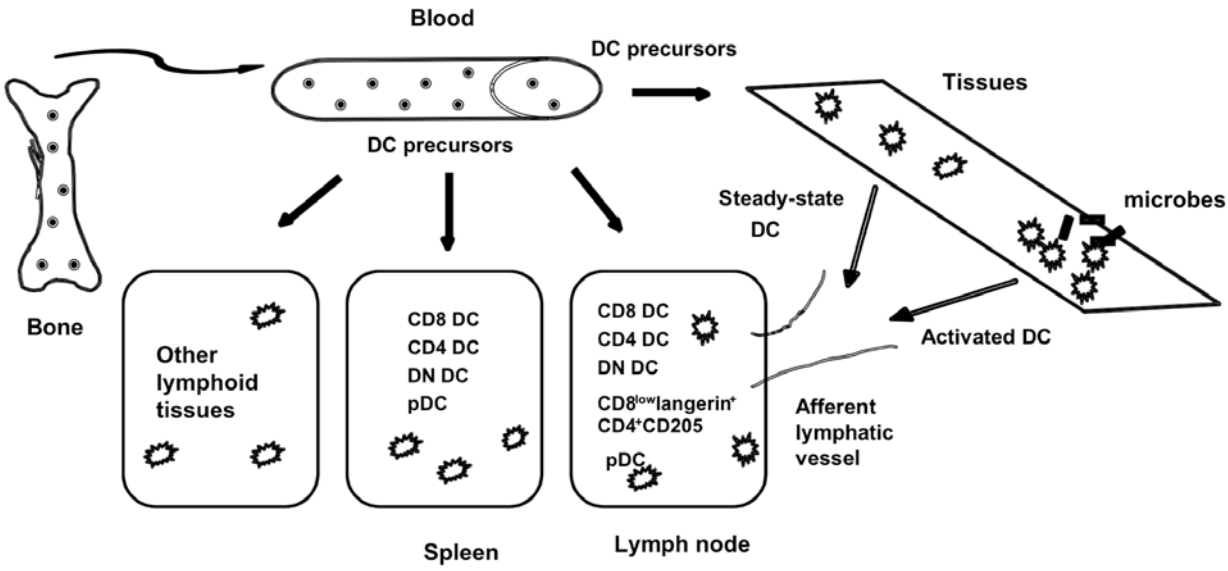

Figure 4 DC migration in vivo.

2.4.1 Skin homing of Langerhans cell (LC) precursors

LC are bone marrow-derived DC that are typically localized in the epidermis and along mucosal surfaces. They represent a critical outpost of the immune system at the interface to the external environment. Langerin, the major constituent of Birbeck granules specialized in the phagosomes of LC is the unique marker for LC. LC replacement is slow under steady-state conditions, but entirely replaced from a local precursor pool, probably from dermal $CD14^+$ cells in contrast to the inflammation-induced replacement of emigrated LC, largely through bone marrow-derived precursors (17, 28).

During trauma and inflammation, immigration of LC precursors from the blood to the epidermis was proposed to depend on a cascade of events, including arrest of rolling monocytes on endothelial cells and transmigration across the endothelial barrier by interaction of adhesion molecules, as well as inflammatory chemokine gradients-guided migration from the dermal to epidermis. During the last step, some of the chemokines have to be activated by matrix metalloproteinases (MMP) and MMP is also involved in the proteolysis proteins in the extra-cellular matrix that has to be crossed by DC precursors to the epidermis (28).

2.4.2 Migration to lymphoid organs

Peripheral DC has the ability to migrate from the tissues to T cell zone in the draining lymph nodes and such is accomplished partially by down-regulation of CCR1 and CCR5 and up-regulation of CCR7 of DC. CCR7 targets DC to lymphatic vessels and lymph nodes via chemokines CCL19 and CCL21. CCR2 is important for DC to translocate into the T cell area, CCR5 may help recruit DC to inflammatory sites and CCR6 appears to be important for positioning DC at epithelial surfaces. CCR7 may increase entry to lymphatics and migration to the T cell area in the LN. Migration is enhanced by leukotriens, released by the DC themselves. Maturation also induces DC to release other chemokines to recruit more monocytes and DC to local sites. Incompletely matured DC induced by TNF-α also up-regulate CCR7 and carry self antigens to LN for the induction of tolerance during steady-state conditions (28-30).

Transfer of antigen in the nodes and T cell priming

After tissue-resident DC have internalized antigen and migrated to the LN, the antigen needs to be presented by MHC or CD1 presenting molecules to the responding T lymphocytes. Although it is not fully clear how DC accomplish T cell priming it has been proposed that there are different DC subsets which sequentially guide the progression of naïve T cells toward fully differentiated effector cells or that each DC subset participates uniquely in the priming of a particular T cell population (29).

The T cell area of lymph nodes, the paracortex, is occupied by three subsets of DC that entered LN from the blood and three via lymph from the tissues. CD4 T cells do not need sustained interaction with one DC to be fully primed, thus priming of T cells in vivo may well require the interplay of a T cell with more than one DC (29). Lately, several lines of evidence have emerged for antigen-transfer between DC under both steady-state conditions and during immune activation. The occurrence of antigen transfer is evident from studies showing the ability of APC to acquire antigen by phagocytosis and then present this antigen by MHC-I, a process called “cross-presentation”. In the LN, a DC interacts with as many as 500 T cells/h, and stable and durable DC-T cell contacts are formed with one DC engaging more than 10 T cells at a time. This interaction contains three distinct phases: initial short encounters of T cells, followed by long-lasting T cell-

DC interaction and leading to cytokine production and up-regulation of activation markers. Finally, T cells dissociate from DC, rapid migrate and proliferate before exiting through the efferent lymphatics (31).

2.4.3 Migration of plasmacytoid DC

pDC are a rare subset of cells present in circulation and in secondary lymphoid organs. They express MHC-II and have the ability to activate T lymphocytes and secret high levels of type I IFN following activation, which is crucial in anti-viral immune responses and in the activation of other leukocytes, such as B and NK cells (32, 33). pDC are normally absent from peripheral tissues and are believed to migrate constitutively from the blood into lymph nodes through high endothelial venules (HEV) (34). This migration is mediated by L-selectin and is increased by an E-selectin mechanism, when LN exposed to inflammatory conditions (35, 36).

2.4.4 CCR7

CCR7 was initially described as a potent chemotactic receptor expressed by migratory leukocytes, including DC. CCR7 has two ligands, CCL19 and CCL21, highly expressed in the stromal cells in the T cell area of lymph nodes. In addition, CCL19 is expressed by mature DC and is presented on the luminal side of the HEV cells. CCL21 is expressed only by afferent lymphatic endothelial cells and HEV. Recent reports have shown additional functions of CCR7, besides chemotaxis. First, CCR7 controls the cyto-architecture (apparition of dendritic protrusions) probably by regulating the organization of the actin cytoskeleton. Reduction of the dendritic protrusions of the DC results in the damping of their antigen-presenting ability. Therefore CCR7-mediated induction of dendritic cytoplasmic extensions may contribute positively to the immune response. CCR7 positively regulates the rate of endocytosis of the mature DC and promotes the survival of DC by delay apoptosis, which would benefit the immune response. In addition, stimulation of CCR7 also increases the migratory speed (motility) of the DC in inducing an increase in the adhesion of these cells to the substrate. Therefore the increase in DC motility controlled by CCR7 may increase the magnitude of the immune response by increasing the number of DC that reach the nodes. Finally, stimulation of CCR7

enhances the mature phenotype of DC leading to the secretion of inflammatory cytokines, increasing the expression of MHC and co-stimulatory molecules and the potential to active naive T cells (37).

2.5 DC functions

2.5.1 T cell homeostasis

The size of the peripheral T cell pool is remarkable stable through life, reflecting precise regulation of cellular survival, proliferation and apoptosis. Independent homeostatic mechanisms regulate the overall size and composition of the naïve and memory T cell-pool. Lymphopenia-induced proliferation (spontaneous proliferation or basal proliferation) describes space-driven expansion of T cells as a compensatory mechanism for restoration of their numbers under conditions of T cell loss. The survival of naïve T cells requires TCR signalling as well as IL-7. Memory T cells are also regulated by homeostatic mechanisms to maintain their overall size and composition. Memory $CD8^+$ T cells can survive in the complete absence of specific antigens requiring IL-15 and CD40 ligation. Lymphoid organ resident DC may play a role in maintaining the naïve T cell pool (38-40).

2.5.2 Tolerance induction

The immune system of vertebrates has evolved to protect against various incoming pathogens by developing an extremely diverse T cell repertoire. However, the generation of a diverse T cell repertoire increases the possibility of developing auto-reactive T cells. To limit self-tissue damage as well as maintaining T cell diversity at the same time, the immune system has therefore developed a number of different mechanisms for tolerance induction (41).

Central tolerance The thymus gland provides a very important initial step in eliminating potentially dangerous T cells during the early T cell development. Positive and negative selection events are considered qualitatively distinct processes depending on thymic compartmentalization and on the cellular context of TCR/MHC interactions. Two cell types present in the thymus are sequentially involved, cortical epithelial cells has the

exclusive capacity to induce positive selection of thymocytes whereas bone marrow-derived DC negatively select auto-reactive cells (41-44).

Peripheral tolerance Peripheral tolerance induction mechanisms are important especially when many tissue proteins are not expressed in the thymus at sufficient levels to induce clonal deletion or tolerization. Several mechanisms of peripheral T cell tolerization involve anergy induction, abortive proliferation/deletion and the induction of immunosuppressive T regulatory cells (Treg). Immature or resting DC, cross-presenting self-antigens in the steady-state, in the absence of inflammation or infection, can induce unresponsiveness to systemic challenge with self-antigens (45-47).

2.5.3 In T priming

DC are critical in generating adaptive immune responses. When a microbe infects, tissue-resident DC not only phenotypically matured by up-regulation of MHC molecules and costimulatory molecules but also functionally mature in priming the adaptive immune responses after migration to the draining LN and presenting the antigens acquired in the periphery.

Reis e Sousa (48) recently argued that the degree of DC maturation largely depends on the quality of the danger signals and that these determine the functional outcome of the effector DC. Main DC effector functions are listed in Figure 5. DC can thus give rise to multiple types of effector DC that instruct distinct T cell fates, including immunity, tolerance and immune deviation. Functionally and phenotypically mature DC can induce Th1 and also Th2 responses, as well as to induce $CD8^+$ cells to differentiate to cytotoxic T lymphocytes.

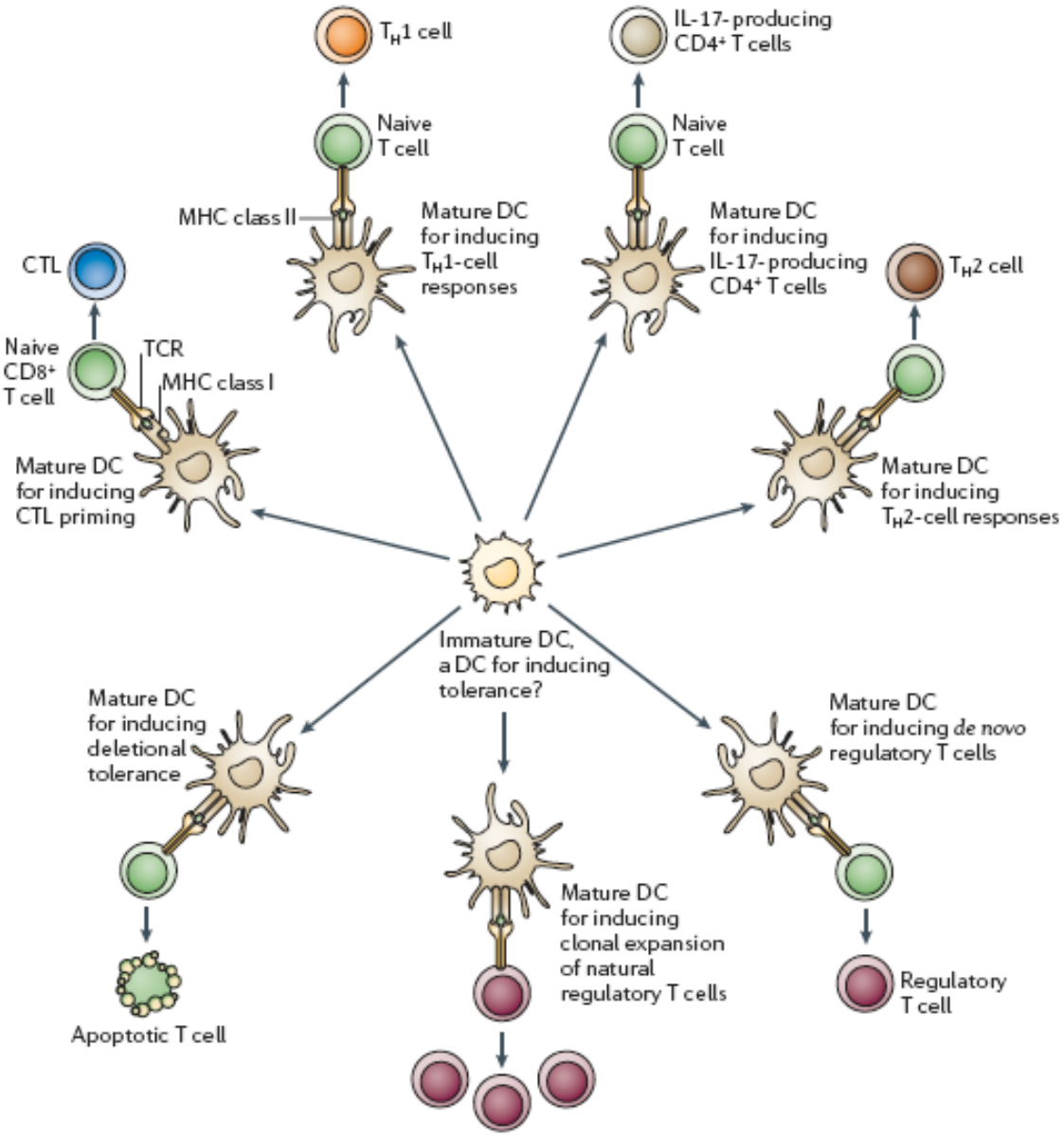

Figure 5 Dendritic cell effector functions (Adapted from Reis e Sousa 2006).

2.5.4 Interaction with innate lymphocytes

DC-NK interaction

Natural killer cells (NK) are believed to limit viremia and tumor growth before the onset of adaptive T and B cell immune responses. NK cells increase in numbers in peripheral blood 3-5 days after infection but subsides at days 5-7. Human PBMC contains around 10% of NK cells. The majority (≥95%) belongs to the cytolytic NK subset, carrying

homing markers for inflamed peripheral sites and perforin to rapidly mediate cytotoxicity. Hypothetically they are the terminally differentiated effectors that carry the whole panel of sophisticated activating and inhibiting receptors to detect allelic HLA loss and can readily lyse these cells (49).

The minor NK subsets (NKT) in blood (≤5%) lack perforin but secrete large amounts of IFN-γ and TNF-β upon activation. In addition, they display homing markers for secondary lymphoid organs, namely CCR7 and CD62L and might perform an immuno-regulatory function in the secondary lymphoid tissues. There are 10 times more NK cells in LN as compared to that in the blood indicating a role for these cells in the LN. In fact, LN NK cells show impressive plasticity in which activation by IL-2 converts these into effector cells with cytolytic function and the expression of the set of activating/inhibiting receptors, similar to the cytolytic NK subsets (49).

NK cell activation by DC Both immature and mature DC can activate and induce the expansion of resting NK cells. Soluble and cell-to-cell contacts have been described for NK cell activation by DC. IL-12, induced by myeloid DC migrated into the LN is mainly implicated in NK cell secretion of IFN-γ. The activated NK cells may provide an early source of IFN-γ required for Th1 polarization. Type I IFN seems to be required for cytolytic activity of NK induced by murine DC. The membrane-bound form of IL-15 expressed on mature DC appears involved in NK cell proliferation and prolonged survival of NK cells. In addition, IFNα is shown to up-regulate the NKG2D ligands MHC-I chain related proteins A/B (MICA/B) on monocyte-derived DC, and these molecules should activate NK cells in cell-cell contact fashion. Also, monocyte-derived DC are recognized and lysed via the NK activating receptor NKp30 through unknown ligands on DC (49-51).

DC activation by NK cells Co-culture of NK and DC leads to DC maturation, production of TNFα and IL-12 and up-regulation of co-stimulatory molecules, such as CD86, relying on either cell contacts or soluble mediators, like TNFα and IFN-γ. In addition, IFN-γ would act as a synergistic or regulatory factor for DC maturation (49-51).

Role for NK cells in DC editing NK cells are highly effective in targeting cells that have down-regulated HLA class I molecules and up-regulated ligands for activating NK receptors due to infection or neoplastic transformation. Immature DC are an exception. NK cells have been shown to sufficiently target autologous immature DC, while in turn DC potentiating NK activation. NK cells remove DC dependent on TRAIL, only when they are in their less-immunogenic immature stage of differentiation. Resistance to NK lysis is achieved by up-regulation of MHC-I, in particularly, HLA-E. Thus NK cells might play an important regulatory role by selectively editing DC during the course of immune responses (49).

DC-NKT interaction NKT cells, unlike the conventional T cells whose receptors are generated via somatic DNA rearrangement, are semi-invariant, consisting of Vα14-Jα18/Vβ8.2 chains in mouse and the homologous Vα24-Jα18/Vβ11 chains in human. Thus they are more like the conserved PRRs of the innate immune system. NKT cells also express a variety of surface receptors that are characteristic of the NK cell lineage. They are activated very early in the immune response and are capable of activating other cell types, including DC (52, 53).

DC presenting the synthetic glycolipid a-galactosylceramide (αGalCer), a marine-sponge-derived glycosphingolipid with many immuno-modulatory activities, on CD1d can activate NKT cells to produce IFN-γ and promotes the resistance to tumors. Activated NKT cells can rapidly induce the full maturation of DC in turn and enhance both $CD4^+$ and $CD8^+$ T cell responses by CD40 ligation (52, 53).

3. Antigen processing and presentation

3.1 Introduction

The conversion of exogenous and endogenous proteins into immunogenic peptides bound to MHC molecules is defined as antigen processing, which involves a series of proteolytic and other events. The cells responsible for antigen processing and presentation are richly diverse, reflecting the antigens and circumstances that the immune system is capable to deal with. Processing and presentation are affected by several factors, including the physical form of the antigens, the site and method of delivery, the nature of the antigen presenting cells (APC) (54). Understanding the cell biology and biochemistry of antigen processing and presentation will help to direct the immune response against various microbes by applying correctly designed vaccines. For instance, in case of $CD8^+$ CTL responses antigens need to be presented in enough numbers on MHC class I molecules expressed on immunogenic APC and in case of antibody responses on MHC class II presenting molecules for the generation of CD4 helper T cells.

The earlier dogma stated that only intracellular, cytoplasmic proteins could be processed for the presentation on MHC-I, whereas MHC-II only presented exogenous proteins internalized into APC. However, as will be further illustrated below, there is an extensively functional cross-over between these pathways with many exogenous antigens capable of generating strong, protective CTL responses. CTL induction by exogenous antigens occur by “cross-priming”, a process which, through a number of different mechanisms, is able to feed immunogenic peptides into MHC-I molecules. However, also non-classical, MHC-I like molecules such as CD1 can present diverse antigens, mostly glycolipids, to a subpopulation of T cells called NKT as they share cellular characteristics with NK cells (55). Glycolipid antigen loading onto CD1 molecules occurs in endolysosomes and most studies of this process have earlier been done in vitro using solid state isolated CD1 (56).

3.2 MHC class I processing and presentation

3.2.1 Classical pathway

Sources of antigens The endogenous source of proteasomal substrates is quite varied, including cytosolic viral products, defective ribosomal products (DRiP) and secretory or membrane proteins retrotranslocated from ER to the cytosol, as shown in Figure 6.

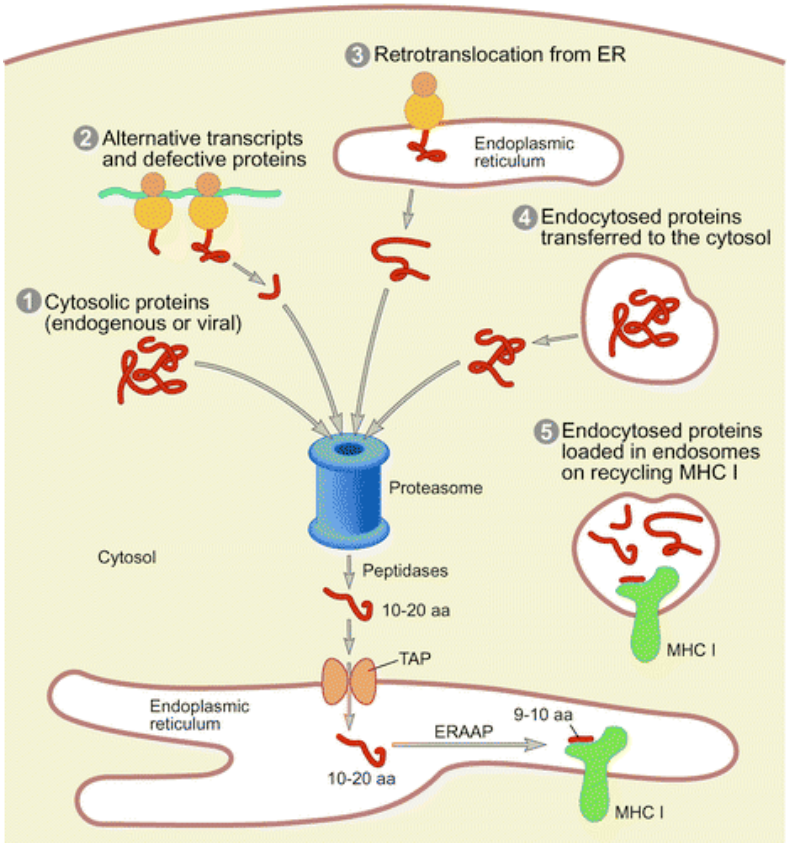

Figure 6 Sources of proteins presented on MHC-I molecules (Adapted from Mellman 2005).

A large proportion of newly synthesized proteins (up to 30%) are known to be ubiquitinated and degraded by the proteasomes shortly after synthesis. Most of these substrates are termed defective ribosomal products (DRiP) and are not functional due to the errors in the process of the protein synthesis. DRiP can also be the result of post-translational mistakes occurring during folding, assembly or intracellular sorting. DRiPs have been proposed to be the major source of self- or viral antigenic peptides for MHC-I presentation. Activated DC can accumulate DRiPs transiently as aggregates, namely DC

aggresome-like-induced structures (DALIS) in the cytosol and are believed to affect the repertoire of endogenous proteins available for proteasomal digestion (57-60). MHC-I ligands are also obtained from "stable" proteins, as evidenced by presentation of several species of post-translationally modified peptides (61-63).

The pathway The classical MHC-I presentation for endogenous proteins comprises several steps: ubiquitination and fragmentation of the proteins by proteasomes in the cytosol, translocation of the generated peptide into the ER via transporter-associated with antigen processing (TAP), formation of the complexes of MHC-I and peptide and display of the MHC-I/p complexes on the cell surface after transportation through the ER-Golgi network, as described in Figure 7.

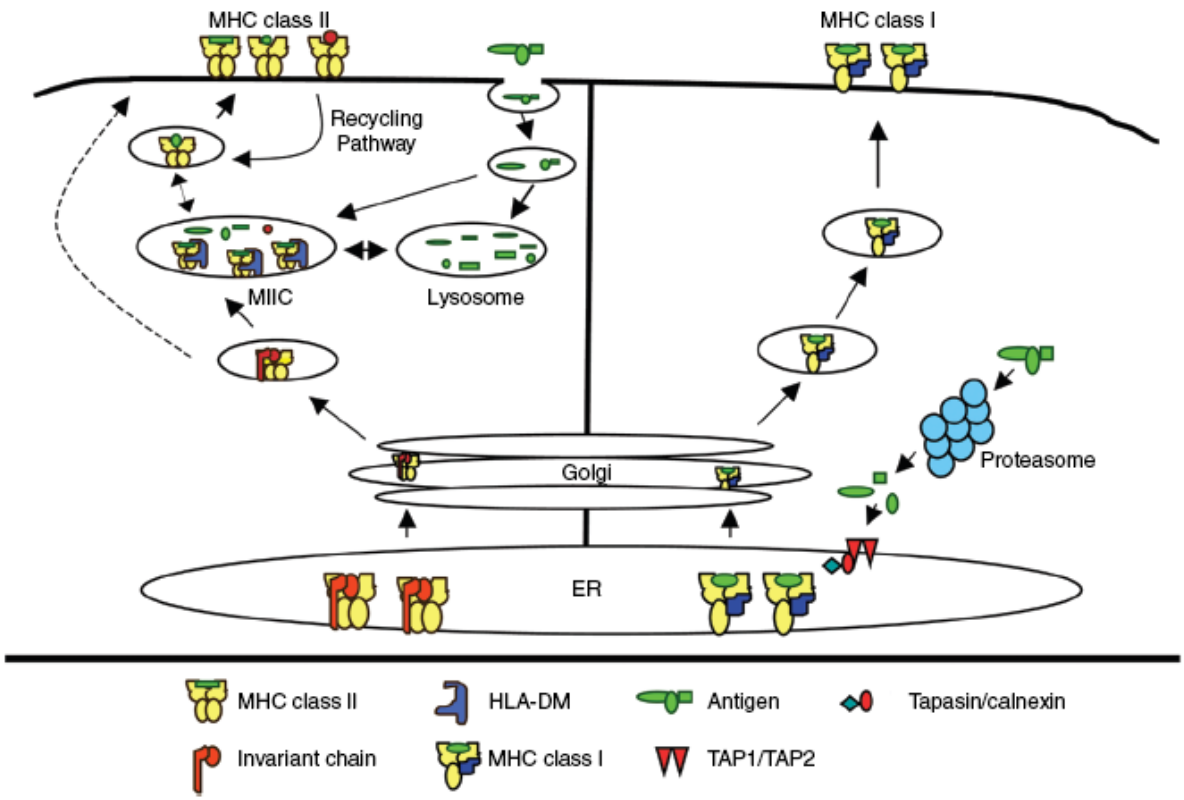

Figure 7 Classical MHC class I and MHC class II presentation pathways (Adapted from Crotzer 2005).

Protein degradation The dynamics of the formation of MHC-I ligands depends on three distinct proteolytic processes. The first step is degradation of proteins into oligopeptides by the 26S proteasomes with the correct size for presentation or extended on their amino-termini. Secondly, aminopeptidases in the cytosol or ER trim the N-extended precursors into the optimal length (8-10aa) for binding to MHC-I molecules. The third process is the destruction of proteasomal products by endo- and exo-peptidases that eventually allow the recycling of amino acids back to protein synthesis. The third process is very rapid in vivo so peptides somehow must escape this fate to become available for MHC-I binding (64).

The 26S proteasomes The immune system has not evolved its own specialized proteolytic mechanisms but utilizes the ancient catabolic pathways that turn over proteins in all cells. The 26S proteasome is an ATP-dependent proteolytic complex, found in the cytoplasm and nucleus (64-68). It is a major cell constituent composing up to 2% of cellular proteins and essential for cell survival. The 26S proteasome is a complex composed of a 20S core particle and one or two 19S regulatory particles. Proteins are degraded within the cylinder-like 20S particles. The three proteolytic sites inside the 20S protesomes function together in an ordered mechanism in degrading substrates, but differ in specificity for different amino acid sequences, referred to as (1) chemotrypsin-like site cleaves preferentially on the carboxylic side of hydrophobic amino acids, (2) trypsin-like site cleaves after basic residues and (3) peptidylglutamyl peptide hydrolase (PGPH) cleaves primarily after acidic residues (69-72).

ER-associated protein degradation Incompletely assembled complexes of ER membrane proteins or misfolded integral membrane proteins in the ER are subject to degradation before they reach the cell surface. These abnormal secretory proteins are translocated into the cytoplasm from ER and degraded by the proteasome pathway. ER also contains signal peptidases and aminopeptidases but clearly lack carboxypeptidases activity, like the cytoplasm (72-75).

Transporter-associated with antigen processing (TAP) TAP is central component in the classical MHC-I dependent antigen presentation. TAP translocates peptides mainly derived from the proteasomal degradation from the cytosol into the lumen of ER, where these peptides are loaded onto MHC-I molecules. TAP belongs to the family of ATP-binding cassette (ABC) transporters, which translocates a large variety of substrates across the membranes driven by ATP hydrolysis. TAP forms a heterodimer consisting of TAP1 and TAP2, with 10 and 9 trans-membrane helices, respectively. Both subunits are essential and sufficient for peptide transport. TAP is localized in the ER and *cis*-Golgi. Each subunit contains a trans-membrane domain (TMD), followed by a cytosolic nucleotide-binding domain (NBD). The TMDs comprise the peptide binding pocket and the translocation pathway for the substrate. The core-domain in the TMD is essential for the peptide-binding and transport, whereas the N-terminal region (N-domain) is responsible for the tapasin binding and the assembly of the peptide-loading complex. The transport cycle is a multi-step process composed of ATP and peptide binding, ATP hydrolysis and peptide translocation. Peptide binding follows two steps reaction with a fast association, preceding a slow conformational rearrangement. TAP binds and transports efficiently most peptides with a length of 8-16 and 8-12 amino acids. Peptides transported by TAP into the ER are subsequently trimmed by amino exo-peptidases in the ER (76-78).

MHC-I assembly and peptide loading (formation of MHC-I/p complex) in the ER
The pathway by which MHC-I acquire MHC-I ligands does not only rely on the relatively inefficient mass transfer from the cytosol, but involves the interplay of the multiple ER-resident chaperones which facilitate the MHC-I loading following transfer of the peptides from cytosol to ER lumen (Figure 8). Initially, nascent MHC-I heavy chain (hc) is targeted to ER and stabilized by the interacting with the ER chaperones Grp78 and calnexin (membrane protein), which are involved in the insertion of the newly synthesized, translocated hc polypeptide into the lumen of ER and the stabilization of it. Once β_2-m associates with the hc, calnexin is exchanged for calreticulin (soluble protein), another ER-resident chaperone because of the conformational changes. The association of MHC-I heterodimer (hc and β_2-m) with calreticulin recruits other ER-resident

chaperones tapasin and ERp57 into the loading complex, which may help the stabilization and folding of MHC-I. ERp57 is involved in assuring the appropriate disulfide bond formation in the MHC-I molecules and prolonged association of this protein which may facilitate the peptide binding in the MHC-I binding cleft. Tapasin is a glycoprotein that bridges the peptide receptive MHC-I heterodimer to the transporter-associated with antigen processing (TAP). Co-localization of these complexes facilities the peptide loading and also stabilizes the peptide receptive state, independent of the TAP association. The MHC-I assembly and MHC-I loading see Figure 8 (79-81).

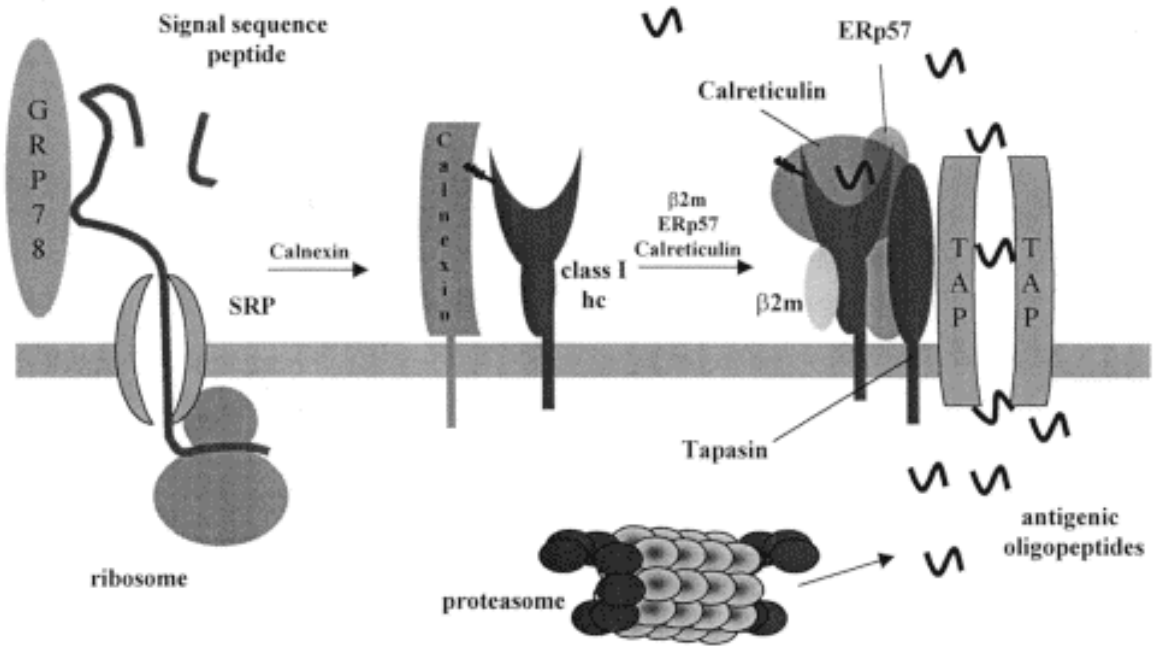

Figure 8 MHC class I assembly in the ER (Adapted from Purcell 2000).

3.2.2 Cross-presentation.

Most cell types in the body express MHC-I molecules and these molecules can be loaded with peptides generated in the cytosol from endogenous antigens. However, exogenous proteins can also be presented by MHC-I molecules, in a process that has been termed "cross-presentation"(82). The exogenous antigens can either be soluble, bound to serum Ig, cell-associated, heat shock proteins or exosomes with the functional consequence of their MHC-I presentation ranging from maintaining tolerance (cross-tolerance) to

inducing immune responses (cross-priming). Cross-presentation probably also contributes to the maintenance of the peripheral naive T cell pool (40).

Mechanisms in cross-presentation Cross presentation may occur by different mechanisms. Some proteins can traverse the membrane of uptake vesicles and access the cytosol, where they are processed as endogenous proteins in a "leakage pathway" (83-86). Alternatively, endocytosed exogenous proteins can be processed within the endocytic compartment and the generated antigenic peptides either bind to membrane-recycling MHC-I molecules or bind to the cell surface after peptide regurgitation (87-97). Finally, ER membrane, equipped with all the classical MHC-I processing machinery may contribute to the formation ER-phagosome and further enable to act as self-sufficient organelles for cross-presentation (Figure 9) (98-102).

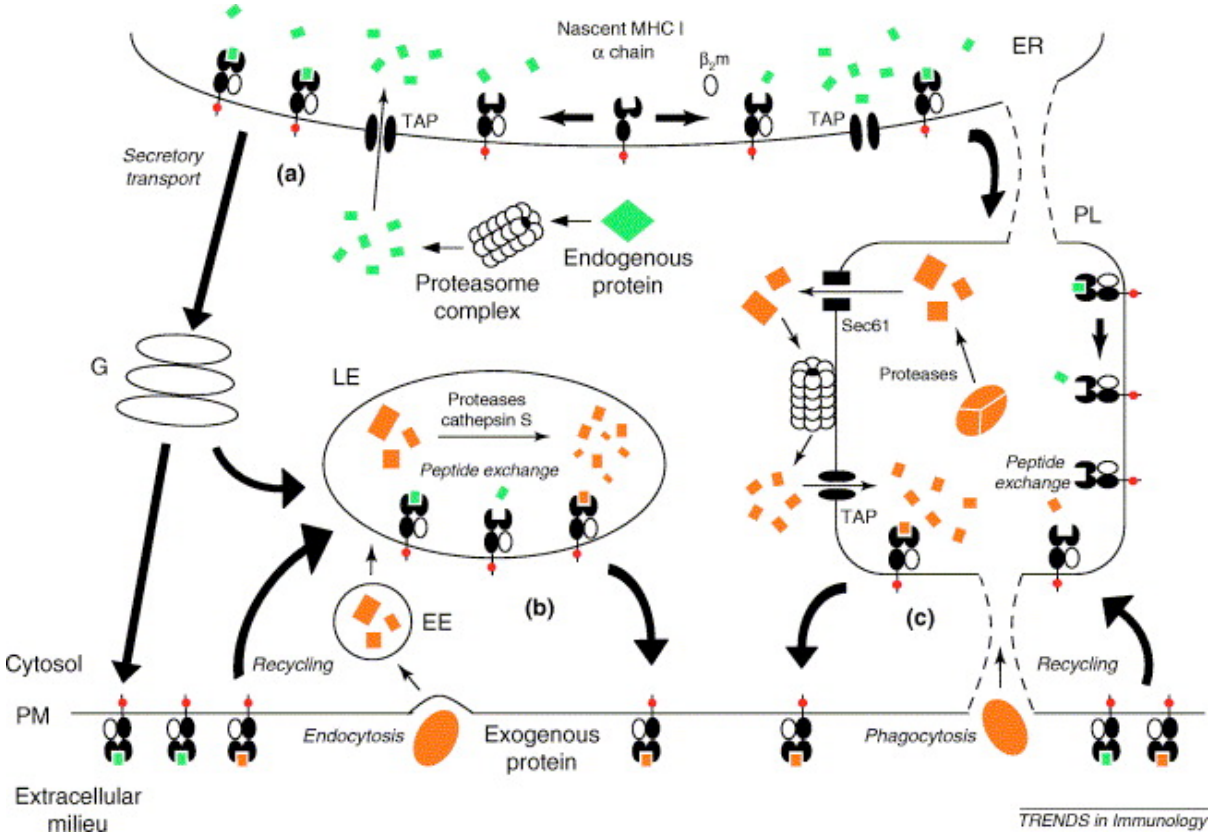

Figure 9 Multiple routes of peptide antigen loading for MHC-I in APC (Adapted from Lizee 2005).

Leakage pathway By leakage pathway exogenous antigens can thus be transferred into the cytosol, where they are further degraded by the proteasomes and loaded onto MHC-I in a TAP-dependent manner. Direct evidence suggested the transport from the endosomes to the cytosol in cross-presentation was first reported using macrophages and then bone marrow-derived DC by tracking the translocation of horseradish peroxidase (HRP) from endosomes to the cytosol (83, 84). It was also demonstrated using immune-complexes, showing a selective membrane transfer mechanism (86). The putative channel that mediated endosome to cytosol transfer could accommodate molecules of a certain size (50-500kDa), as analyzed by using dextrans of variable size (86).

ER-phagosome pathway Recently several groups have independently described that the ER membrane contributes to the formation of phagosomes. The ER-phagosomal compartment in macrophages and DC contains endocytosed antigens, newly synthesized MHC-I molecules, and the chaperone proteins necessarily for the formation of MHC-I/peptide complexes, namely tapasin, ERp57 and TAP. The internalized proteins are translocated into the cytosol by the sec61 transporter for proteasomal degradation and the produced peptides are thereafter transported back into the phagosome by membrane-bound TAP (Figure 9). The Sec61 complex is a heterotrimer that forms the channel that allows newly synthesized secretory or membrane proteins to cross from the cytosol into the ER. This channel can also be used to transfer misfolded polypeptides from the ER to the cytosol for proteasomal degradation, a process termed "dislocation" or "retro-translocation" (103, 104).

Vesicular processing (VP) Endocytosed exogenous antigens can also be processed in the endolysosomal compartment. Evidence for this pathway includes using bacterial-associated OVA (87-90), bead-OVA (91, 92), cell-associated viral antigens (93-95) or even soluble OVA (96). Features of this type of MHC-I processing are TAP-independent, BFA- and lactacystin-resistant, and the latter are the Golgi and proteasomal inhibitor, respectively. MHC-I presentation by this pathway is clearly different from the cytosolic pathway in which proteasome and Golgi are dispensable, suggesting an endolysosomal or an intravesicular compartment exists for this type of processing. The generated/resulted

peptide was shown to bind to MHC-I molecules either within the vesicular compartment (95) or regurgitated to the cell surface and subsequently binds to cell surface MHC-I molecules (87) or both (96), depending the system used (Figure 9). The next question is where the MHC-I molecules in the vesicles come from? In general, MHC-I molecules are not found in the endocytic vesicles of most cells, but it may enter this compartment in phagocytes in several different ways. Studies have indicated that phagocytes constitutively recycle MHC molecules between cell surface and the endocytic compartment (105), secondly MHC-I can also be targeted to the endocytic compartment by Li (106), an endogenous chaperon peptide used to target MHC-II to the same site, and thirdly cell surface newly synthesized MHC-I can be internalized into these vesicles (107) as the invagination of the plasma membrane during phagocytosis.

Recently, It was demonstrated that cysteine protease Cathepsin S (Cat S) in the endolysosomes was involved in this type of processing by showing that purified Cat S could process OVA into SIINFEKL peptide in vitro and further demonstrating that Cat S played a role in generating CTL against two viral epitopes in vivo (91, 108, 109). Another study shows there are endolysosomal targeting motifs in the cytoplasmic tail of MHC-I and mutations in these impair CTL responses. This mechanism is independent of the TAP and the components of the MHC I peptide-loading complex, although for long-term nascent MHC-I is needed for the refill of the MHC-I pool in the endocytic pathway (110, 111).

Endosome to ER pathway This pathway is based on findings that some of the soluble exogenous proteins can be internalized, entered ER and subsequently transferred to the cytosol for degradation by ER-associated degradation (ERAD) This transport process may be inefficient, but in the case of toxins, the internalization and subsequently reaching the cytosol are sufficient to block the majority of the total TAP function (112, 113).

Gap junction pathway Most tissue cells are electrically coupled with their neighbouring cells through gap junctions, the small channels that connect the cytosol of adjacent cells. DC and activated monocytes can establish gap junctions with other cells, including

infected cells and thereby may acquire antigenic fragments for cross-presentation. However, tumor cells usually close their gap junctions and rather live solitary lives, which may partially explain the poor CTL responses (114-116).

Other minor important pathways include the extracellular MHC-I processing by secreted and membrane-associated proteases and peptide-penetration directly into the ER without the help of TAP.

3.3 MHC-II processing and presentation pathway

Formation of MHC-II/peptide complexes is an orchestration of multiple cellular processes, including protein-sorting mechanisms, proteolytic activities and the intervention of chaperones. Many of these mechanisms are regulated in DC in a manner distinct from other APC (54, 117, 118). Major steps in MHC-II processing and presentation are described in Figure 7.

The endocytic pathway Describing the process of MHC-II antigen presentation requires first the description of the subcellular structures, where the formation of MHC-II/p complexes takes place, namely the endocytic pathway (Figure 10).

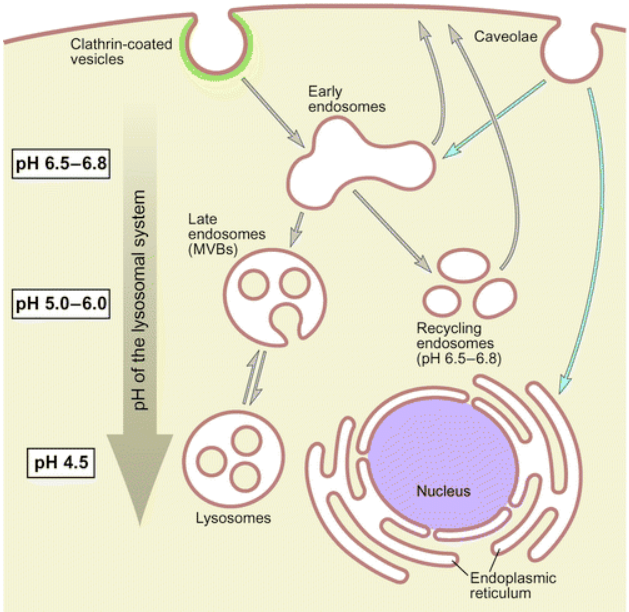

Figure 10 Organelles of the endocytic pathway (Adapted from Mellman 2005).

The endocytic pathway is a complex network spanning from the plasma membrane to the lysosomes. This road is intersected by secondary incoming and outgoing tracks. The incoming tracks represent vesicles originated mostly from the cell surface and the distal components of the Golgi complex, while the exit tracks and vesicles eventually will fuse with the plasma membrane. The contents of the endosomal compartments change gradually along the major track and therefore it is not possible to clarify the exact boundary defining the endocytic pathway. Three major regions are usually distinguished: the early endosomes (EEs), whose limiting membrane and lumen have a similar composition to the plasma membrane and the extra-cellular medium; the late endosomes (LEs) which contain many components found only in the endocytic route; lysosomes, which are considered as the final station of the endocytic pathway, acid and highly proteolytic (54, 117-120).

Endocytosed materials move from predominantly along the EE-LE-lysosomes axis. As a rule, the number of entry and exit tracks along the major axis is most abundant in the EEs and least in the lysosomes. The sorting mechanisms, which decide how any given protein accesses and how it leaves the endocytic route are still poorly understood. For example, some receptor cycles continuously shuttle from plasma membrane to the EE and back. The DEC205 receptor transports antigens bound at the plasma membrane to LE and lysosomes releases them there and then returns back to the cell surface (121). Endosomal proteases are delivered to the endocytic rout directly from the Golgi and largely retained in the endocytic compartments (117, 122).

Two features of the endocytic route are important in the context of antigen presentation. First, the sorting mechanisms allow for some degree of “leakiness” so that for example, the cathepsins can be both secreted and delivered to endosomal compartments (123, 124). Second, the pathway is not always linear; rather it is possible that some compartments branch off becoming distinct reservoirs for endocytosed materials or parallel subpathway with different dynamics and composition than those of the main EE-LE-lysosomes (117).

Antigen degradation Whatever mechanism is used for the internalization, antigens progress along the EEs-LEs-lysosomes axis, where they are exposed to an increasingly more acidic, reducing, and proteolytic environment (125, 126). The result is a gradual release of polypeptides along the entire endocytic pathway, a few of which will contain the correct amino acid sequence that permit them to fit into the binding cleft in MHC-II molecules for $CD4^+$ T cell recognition (127, 128).

Formation of MHC-II/p complexes Newly synthesized MHC-II αβ dimers are translocated into the endoplasmic reticulum (ER) and further transported to the endosomal compartments.

Li processing MHC-II molecules are synthesized in an inactive form in which their peptide-binding cleft is occupied by the chaperone invariant chain Li. Li contains in its cytosolic portion a sorting motif that is recognized in the trans-Golgi network as a signal to haul the dimer-li complex out of the secretory pathway and deliver it into the EE/LE regions of the endocytic pathway. Once the MHC-II-Li complexes reach the endocytic pathway, Li is eliminated by Cathepsin S (Cat S) after its cleaving Li into the final peptide fragment CLIP residing in the MHC-II cleft. Thereafter, CLIP has to be substituted for antigenic peptide, a reaction involved the chaperones H-2DM, which interacts transiently with MHC-II-CLIP, destabilizing the complex and facilitating the release of CLIP. H-2DM not only assists in the removal of CLIP, it also acts as a peptide editor, promoting the exchange of low affinity peptides for high-affinity peptides. The newly formed MHC-II/p complexes can then be sorted from the endocytic route toward the plasma membrane in transport vesicles (117).

MHC-II/p complex formation A key question in the MH-II processing remains unsolved: are peptides generated first and then bind to MHC-II molecules as that of MHC-I pathway or peptides generated after the intact antigen bind to MHC-II (Figure 11). The first model is likely presumed to be prevalent but it is problematic as 10-15aa peptides rarely accumulate in the terminal degradative environment of lysosomes. The alternative model proposes that epitopes could be protected from destruction by binding

to MHC-II molecules. Also the open binding groove in MHC-II allows the binding to whole or long polypeptides and the protruding ends could be trimmed by endo- and exo-peptidases while the peptides in the binding groove are protected.

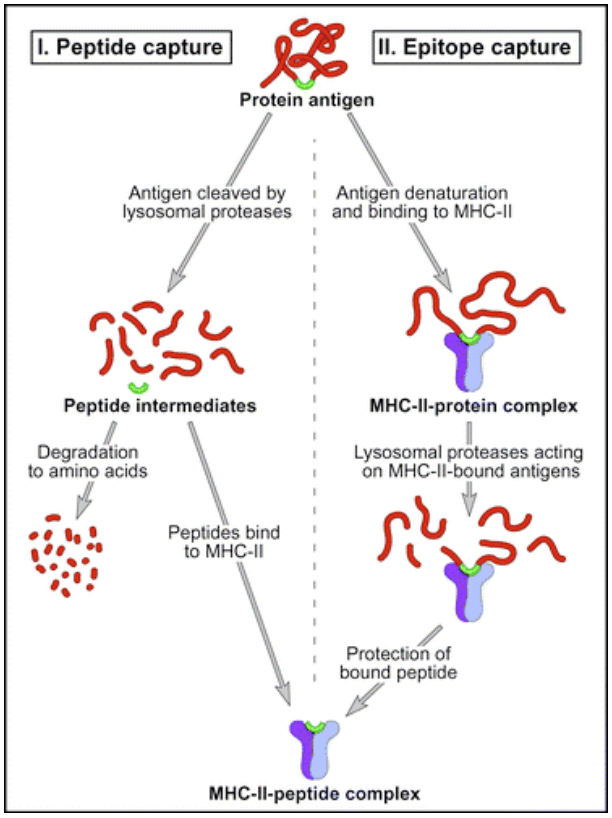

Figure 11 Possible pathways for generation of MHC-II/p complexes (Adapted from Mellman 2005).

Control of MHC-II processing pathway Evidence indicates the formation of MHC-II/p complexes by DC can be regulated under physiological conditions suggesting that lysosomal proteolysis could be potentially regulated independent of regulation of co-stimulation, creating putative immature but active tolerogenic DC (47).

Control of antigen degradation has been proposed as one mechanism used by DC to regulate MHC-II presentation. The increased proteolytic activity during maturation is not caused by an augmented protease expression, rather by the further acidification of the endolysosomal compartments. This is supported by the findings that the vacuolar H^+-

ATPase pump responsible for lysosomal acidification, was recruited from the cytosol to the endosomal compartments during cellular maturation.

The acidification of the endolysosomal compartments can modulate antigen degradation and peptide loading in several ways including favouring the binding of peptides or large fragments to MHC-II, dissociation of Li and peptide editing by H-2DM.

3.4 Mechanisms of exogenous antigen capture

The simplest way of loading peptides on MHC molecules is by direct binding of extra-cellular peptides to MHC molecules on the cell surface, mostly used in epitope-mapping studies and in clinical trials. APC can sometimes present peptides generated and released by neighbouring cells by peptide exchange. Formation of MHC/p complexes directly at the cell surface may lead to conformations and specificities different from those formed intracellularly. Conversion of internalized antigens into MHC/p complexes is more prevalent and efficient at intracellular sites. Ingestion of microbes is generally accompanied by inflammatory responses, whereas the opposite may occur after uptake of apoptotic or necrotic cells. In DC, endocytosis is upregulated shortly after the maturation process is induced but subsequently downregulated. Antigens can be endocytosed by a variety of mechanisms (Figure 12) (54, 117).

Phagocytosis needs recruiting the actin cytoskeleton to mold the plasma membrane around the phagocytic particles (phagosomes). **Macropinocytosis** also requires actin to form vesicles (micropinosomes) for the large portion of extracellular medium engulfed. **Micropinocytosis** does not require actin polymerization for vesicle formation (pinosome) but requires the recruitment of the cytosolic protein clathrin to generate clathrin-coated pits. Finally, **receptor-mediated endocytosis** consists of the internalization of molecules by membrane–bound receptors, many of which also trigger the formation of clathrin-coated pinosomes. Uptake of materials by macro- or micropinocytosis is often referred to as fluid-phase endocytosis to indicate that it is non-specific, rather than being triggered by particular molecular cues. DC are highly efficient at all forms of endocytosis in their immature state (54, 117).

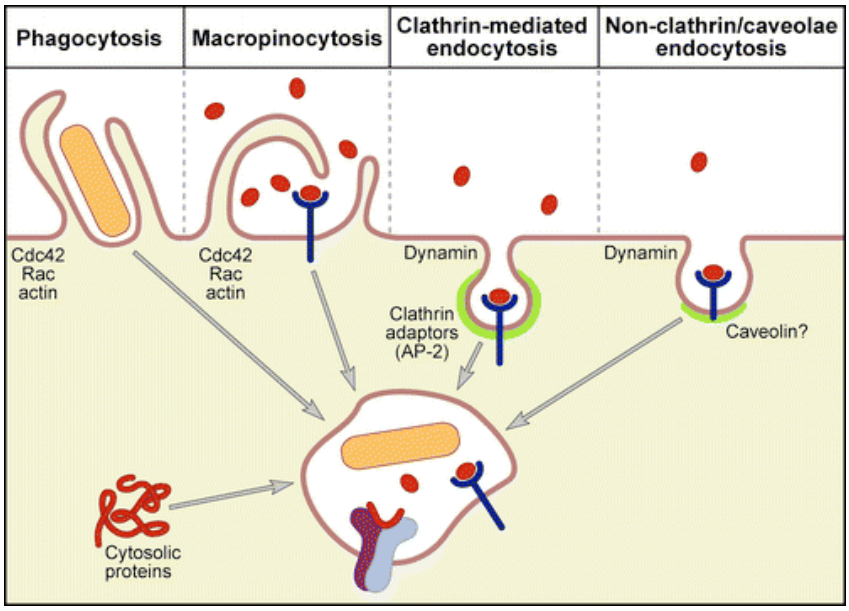

Figure 12 Types of endocytosis used for antigen accumulation (Adapted from mellman 2005).

One of the most common ways of studying antigen presentation ex vivo has been to expose APC to a large amount of soluble antigens such as OVA. Immature DC and macrophages internalize the bulk of soluble antigens via macropinocytosis. The impact of antigen uptake via macropinocytosis in vivo is not fully clear yet, although it may apply to those cells encountering antigens present in body fluids. Furthermore, pathogens release few soluble antigens, as most antigens are associated with membranes, cell walls, or cytoplasmic compartments (54, 117).

The uptake of particulate antigens is a prevalent form of antigen uptake in vivo for both pathogen-derived, cell-associated antigens or artificial beads. Phagocytosis is probably the first pathway associated with the host defense. A number of surface receptors recognizing ligands on the surface of microbes contribute to the pathway, including FcRs, complement receptors and a variety of lectin receptors. Sometimes microbes can even actively promote their own entry into cells. The engulfment of apoptotic cells from infected cells provides antigenic materials that can be readily converted to MHC/p

complexes. Ingestion of apoptotic cells bearing self-antigen is also important for the maintenance of tolerance during steady-state conditions (54, 117).

The immune system thus uses a broad range of cell surface receptors to capture extra-cellular materials with two important consequences. Firstly, the quantitative advantages make cells more efficient to present antigens at much lower concentrations. Secondly, qualitative aspects contribute to functional diversity among different APC. For example, DEC205, a lectin-like receptor preferentially expressed by DC that is internalized by clathrin-coated vesicles. Targeting antigens to DEC 205 by using monoclonal antibody against DCE205 results in 100-1000 fold more efficient in antigen capture and presentation than soluble antigen, subsequently inducing either tolerogenic responses (under non-inflammatory conditions) or strong immunity of CD4 or CD8 cells under activating conditions (simultaneous CD40L treatment) (54, 117).

3.5 CD1 as an antigen presenting molecule.

3.5.1 Introduction

CD1 proteins are a third family of antigen presenting molecules that can bind bacterial and autologous lipid antigens for presentation to NKT cells. CD1 molecules are glycoproteins composed of a heavy chain non-covalently linked to β-2m, similar to MHC-I molecules. Unlike MHC-I, CD1 molecules have evolved hydrophobic channels for the binding of alkyl chains and also have cytoplasmic tails that target them to distinct endocytic compartments (55, 129) (Figure 13).

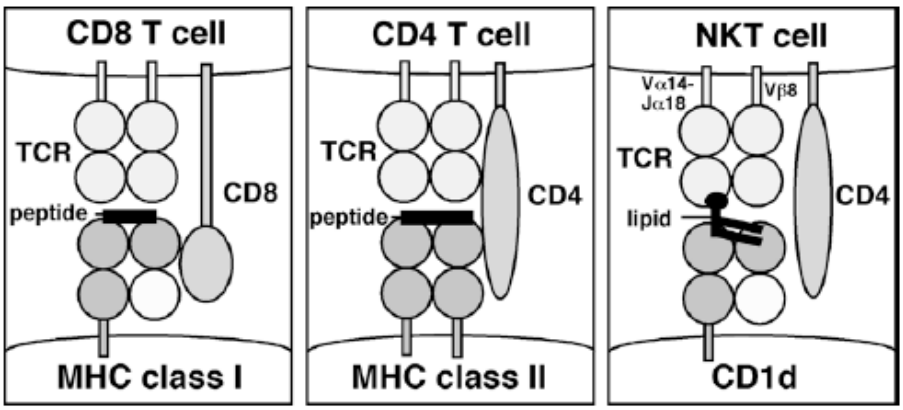

Figure 13 Display of MHC and CD1 molecules (Adapted from Van Kaer 2004).

CD1a, CD1b and CD1c (group 1) molecules present microbial fatty acids, glycolipids, phospholipids and lipopeptides by anchoring the alkyl chains of the ligand within their hydrophobic binding grooves, positioning the polar head group or hydrophilic cap of the bound ligand at, or near the opening of the groove for the recognization by NKT cell receptor. CD1d (group 2) molecules are believed to present self-lipid antigens, including sphingolipids and diacylglycerols, as well as an unusual sponge-derived ceramide (αGalCer) with the similar mechanism (55, 129). CD1a, CD1b and CD1c-restricted TCRs are extensively germline-derived with marked junctional diversity, while CD1d-restricted T cells include both with an invariant TCR (Vα14-Jα18 in mice, Vα24-Jα18 in humans) and those with extensive α/βTCR diversity (130, 131).

3.5.2 CD1 assembly, trafficking and antigen sampling

Following translocation into the endoplasmic reticulum (ER), CD1d forms a temporarily complex with calnexin and calreticulin. The temporary complex in turn recruits ERp57, which catalyzes disulfide bonds in the CD1d heavy chain. Fully oxidized CD1d disassociates from the complex, associates with β_2-m and enters the secretory pathway. CD1b strictly requires the association with β_2-m in order to exit from the ER to the cell surface, while a portion of CD1d can exit without the light chain. Self-lipids such as GPI, PI or PtdIns may be loaded onto CD1 during assembly in ER and trafficing through the secretory pathway and endocytic pathways. These self-lipids are rarely antigenic and appear to act as chaperones that aid in the assembly of nascent CD1d molecules (Figure 14).

After synthesis and assembly in the ER, majority of CD1b and CD1d are transported to the plasma membrane and thereafter reinternalized. Each isoform differentially traffics through the endocytic pathway, where preloaded self-lipid can be exchanged for endosome-resided self- or microbe-derived lipids before transporting to the plasma membrane (130, 131). CD1b and CD1d are specifically targeted to the late endosomes and lysosomes through the interaction of a tyrosine-based motif in the cytoplasmic tails and the adaptor protein AP-3. Functionally AP-3-dependent trafficking is essential for mouse CD1d presentation of endosome-derived lipids to Vα14-CD1d restricted NKT

cells, but is dispensable for human Vα24 CD1d-restricted and mouse NKT with diverse TCRs (130, 131).

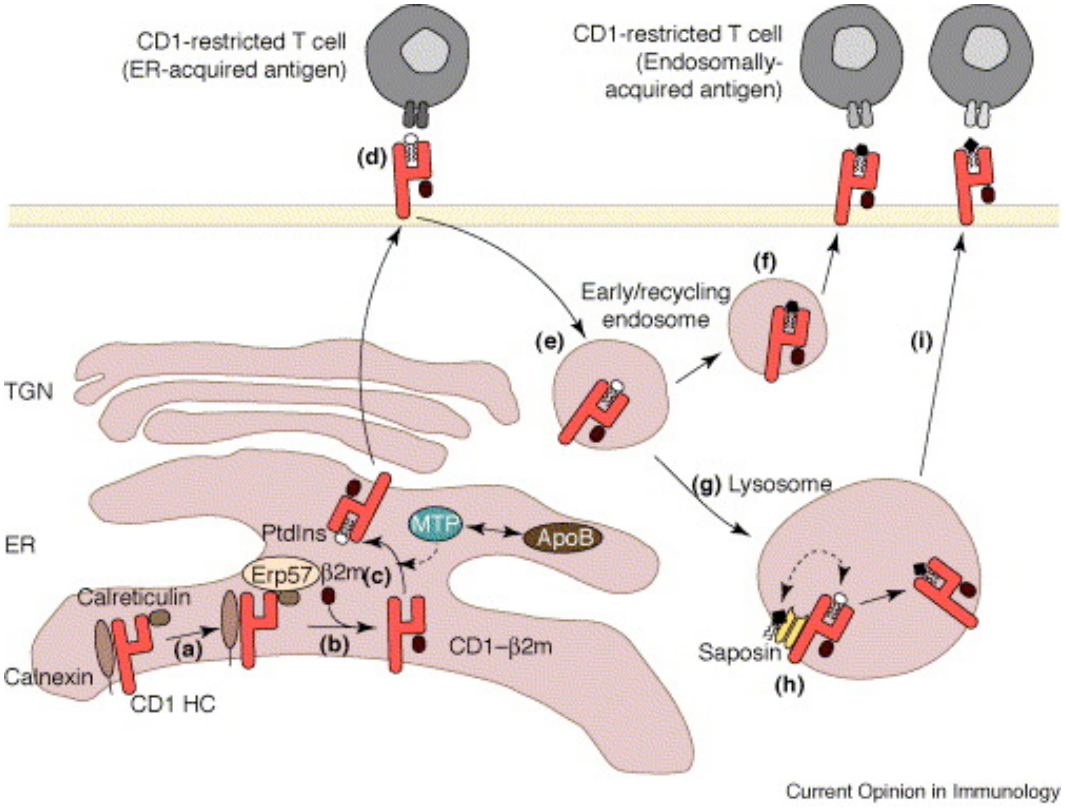

Figure 14 CD1 assembly and traffic (Brenner 2005).

Earlier methods described by Kronenberg (56) in measuring CD1d binding were conducted using plate-bound soluble CD1d molecules and these molecules were purified from insect tissues. As CD1 molecules are quite hydrophobic and easily get aggregated, they immobilized the purified CD1 molecules by attaching them to microplates and loaded the molecules with known ligands. Thereafter the complexes were determined by hybridomas. Indeed, glycolipids (GLs) are also hydrophobic and easily stuck to tissues, so it is difficult to deliver a vaccination just by introducing GLs. It is reasonable to think that GLs or lipids are loaded onto CD1-expressing DC in vivo before the induction of NKT function. However no data have shown the direct binding of GLs to CD1d molecules in living cells, especially DC.

Accessory molecules in CD1 presentation (quality control)

MTP (microsomal triglyceride transfer protein) is an ER-resident protein able to transfer lipids between vesicles, especially in the loading of self-lipids onto the CD1d-binding groove in the ER. It is also involved in the assembly and secretion of apolipoprotein B (ApoB). Deletion of MTP alters CD1d trafficing, decreases surface expression of CD1d and abrogates CD1d presentation to both NKT and *i*NKT.

SAPs (sphingolipid activator proteins) are small cystein-rich proteins that have an essential role in the degradation of glycosphingolipids in lysosomes and lipid-transfer to CD1. Four SAPs (saposins A-D) are derived from the common precursor pro-saposin, but differ in their mechanical action. SAP-deficiency results in lysosomal storage disorder and diminishment of lipid presentation. Thymocytes from the deficient mice fail to activate auto-reactive *i*NKT or even mice completely lack NKT cells, suggesting a role of saposins in loading lipids in the thymus for the generation of positive selection in mice (130, 131).

Adapter proteins (AP) The AP family, AP-1, -2, -3 and -4 recognizes the tyrosine-based sorting motifs in the cytoplasmic tails and targets proteins to specific subcellular compartments. **AP-2** is important for the internalization of proteins from the cell surface, such as hCD1d and mCD1d. **AP-3** is important for late endosomes and lysosomes targeting, in the case of hCD1b and mCD1d (130, 131).

It should be noted that most newly synthesized MHC-II move directly for the Golgi network to endosomes directed by Li, while CD1b and CD1d move rapidly to the cell surface after exit from the Golgi and recycle back to the endosomes. Secondly, MHC-II trafficking and presentation are not influenced by the absence of AP-3, indicating MHC-II and CD1 employed different routes for the entry of the endosomal compartments.

3.5.3 Carbohydrate antigen processing for CD1 presentation

Proteolytic processing in the endocytic compartment is crucial for MHC-II presentation, for the degradation of Li and the processing of extracellular proteins. It is likely that there

are analogous processes of lysosomal carbohydrate processing for CD1 presentation. **α-galactosidase A**, located in lysosomes removes the outer sugar to yield the monosaccharide from certain α-GalCer analogues for the recognition of Vα14i NKT cells. α-GalCer is a glycolipid extracted from the marine sponge Agelas *mauritianus*. **β-hexosaminidase**, a lysosomal enzyme, removes β-linked N-acetylgalactosamine (GalNAc) residues on glycosphingolipids, ganglio-, globo- and lacto-series to expose iGb3, an endogenous mammalian antigen, recognized by Vα14i NKT. Mice deficient in this enzyme do not have Vα14i NKT cells and also unable to stimulate the corresponding hybridoma, indicating iGb3 is likely required for the positive section of Vα14iNKT cells in the thymus.

3.5.4 NKT Cells

NKT cells have the ability to rapidly secrete a variety of cytokines including IL-4, IFN-γ and TNF-α within a few hours after activation. The activation of NKT cells can lead to either suppression or stimulation of immune events making them potential regulators of the immune responses against certain microbes, tumors, self-tolerance and autoimmune diseases. In addition, NKT cells can directly cytotoxic for infected and tumor cells (52, 53).

NKT cells represent a unique lymphocyte lineage and were originally characterized in mice as cells coexpressed a rearranged T cell antigen receptor (TCR), the CD3 complex as well as several receptors that were first identified on NK cells, such as NK1.1 (C type lectin) and various Ly49 molecules. However, sometimes NKT cells do not express NK1.1 and also conventional $CD8^+$ T cells can acquire NK1.1 during activation. Invariant NKT cells express a Vα14-Jα18 rearrangement and respond to αGalCer presented by CD1d molecules. Humans have a homologous population of T cells with an invariant Vα24 rearrangement, namely (Vα24-Jα18 NKT cells). These cells are known by several names including type I NKT cells, invariant (*i*) NKT cells and Vα14i NKT cells (52, 53).

Vα14*i* NKT cells arise in the thymus and do not reach significant levels until three weeks after birth. Vα14*i* NKT cell development requires the expression of NF-Kβ1 (p50) in a cell-autonomous manner and differentiation requires the transcription factor T-bet. Adaptor protein AP-3 and saponins are also required for the Vα14*i* NKT cell development. In contrast to conventional thymocytes, most of the Vα14*i* NKT cells in the thymus are part of a mature, immune competent population, capable of producing IL-4 and INF-γ immediately after TCR engagement (52, 53).

Two models have been proposed regarding the Vα14*i* NKT cell development. Instructional model proposes that Vα14*i* NKT cells are developed from double-positive thymocytes and further instructed by endogenous lipids presented by CD1d. Pre-commitment model suggests a subset of thymocyte is pre-committed to become Vα14*i* NKT cells before antigen receptor rearrangement (52, 53).

Positive selection is mediated by CD1d-expressing bone marrow-derived cells rather than by MHC-expressing cortical epithelial cells as for the selection of the conventional T cells. **A**utoreactive Vα14*i* NKT ells also undergo negative selection in thymus (52, 53). IL-15 plays the dominant role in governing the homeostasis of these cells, including survival, turnover and lymphopenia-induced or homeostastic proliferation. Lymphopenia-induced proliferation does not require CD1d expression. The requirement of IL-15 and independence of TCR engagement for their proliferation make these cells more similar to conventional memory T cells (52, 53).

Vα14*i* NKT cells are most prevalent in the thymus, spleen, liver and bone marrow, but less abundant in lymph nodes and rare in the intestinal mucosa. They express a set of chemokine receptors consistent with a tissue-seeking function of these, with relatively little expression of CCR7. Mouse Vα14*i* NKT cells can migrate to the sites of inflammation in the lung, liver and spleen. Activated Vα14*i* NKT cells have both perforin-dependent and FasL-dependent cytotoxic functions, which are dependent on TCR engagement. Activated NKT cells can show a strong anti-tumor effect in some systems and can also provide an adjuvant effect during a vaccination process.

Understanding the mechanisms behind these effects may guide therapeutic strategies based on NKT cell activation (52, 53).

4. Adenosine (Ado) effects on immunity and inflammation

4.1 Introduction

The purine nucleoside adenosine (Ado) is normally present in body fluids at low concentrations (20-300 nM), but these levels rise markedly during conditions such as hypoxia, inflammation and tissue damage (132-134). Ado is formed by breakdown of ATP and other adenine nucleotides intra- and extracellularly, acts on four, evolutionarily conserved and pharmacologically well-characterized G protein-linked receptors and regulates several physiological processes (132, 134, 135).

4.2 Formation of endogenous Ado

Under normal condition, Ado is continuously formed intra- as well as extracellularly. The intracellular production results from either the intracellular breakdown of AMP (Adenosine 5'-monophosphate) or the hydrolysis of S-adenosyl-homocysteine by a S-adenosyl-homocysteine hydrolase. AMP is formed during energy production from ADP and ATP. At physiological conditions, AMP is at low levels in cells and any AMP that accumulates diffuse out of the cell down its concentration gradient or is converted to ADP or ATP. In addition, Ado is unstable and its half-life (1.5sec) is limited by deamination (Ado deaminase or ADA) or cellular reuptake (Figure 15) (132, 134, 135).

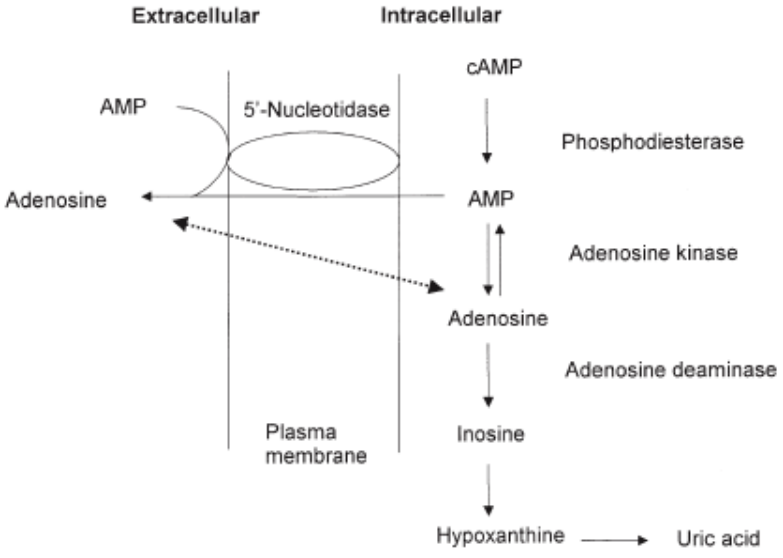

Figure 15 Diagram shows the metabolism of adenosine (Adapted from Livingston 2004).

4.3 Adenosine receptors

There are four established Ado receptors cloned, namely A_1, A_{2A}, A_{2B} and A_3 (Figure 16) (135).

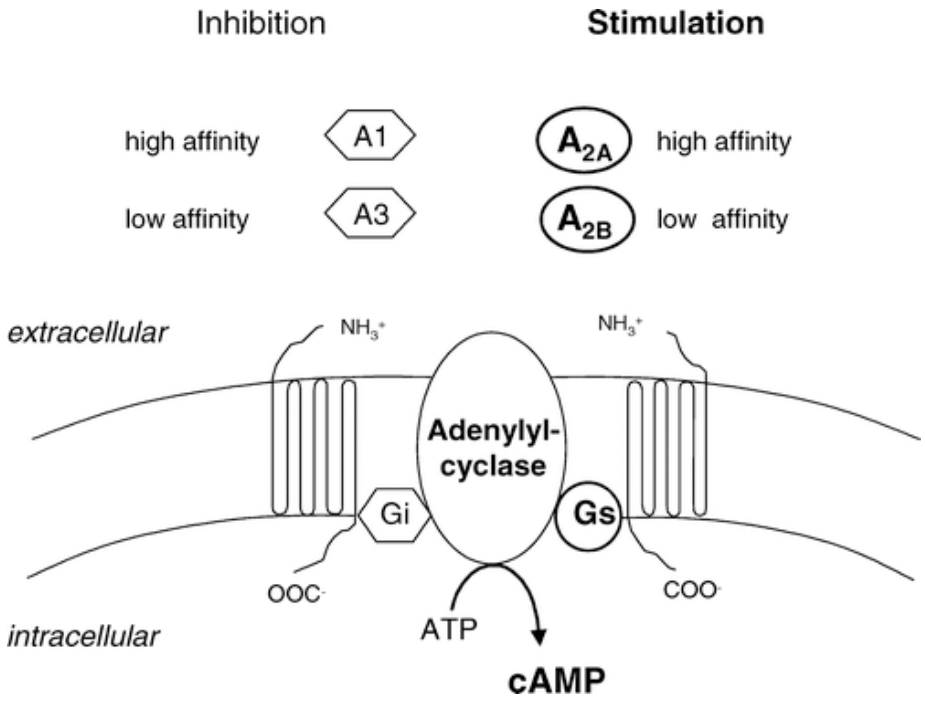

Figure 16 Ado receptors and their effect on cAMP (Adapted from Sitkovsky 2004).

The **A_1** receptor has a very high affinity for Ado and for some Ado analogues. A_1 receptor is coupled to G_i proteins mediating the inhibition of adenylyl cyclase. A_1 receptor is found in heart muscle, central nervous system and inflammatory cells. A_1 receptor activation can also inhibit Ca^{2+} channels. As A_1 and A_3 receptors are coupled to G_i, G_0 and G_q proteins, they not only mediate inhibition of adenylyl cyclase but also activation of phospholipase C. Phospholipase C cleaves phosphoinositide into diacylglycerol and inositol, the latter mobilizes Ca from intracellular stores. Activation of pertussis toxin-sensitive G_i protein induces an intracellular Ca^{2+} transient and actin reorganization.

The **A_{2A}** receptor activates adenylate cyclase resulting in the elevation of intracellular cAMP. A_{2A} receptors are more widely distributed than A_1 receptors and are found in nerve terminals, mast cells, airways, smooth muscle tissue and circulating leukocytes. A_2

receptors are subdivided into the A_{2A} and A_{2B} receptors, based on high and low affinity for Ado, respectively.

A_3 receptor activation leads to the inhibition of adenylyl cyclase, but directly stimulates phospholipases C and D and also influx of calcium and its release from intracellular stores.

4.4 Immune regulation by Ado

Ado receptors (ARs) are expressed on many cell types, including macrophages and DC and are important for both innate and adaptive immune responses. ARs expressed on these cells regulate events such as cellular trafficking, adhesion, chemotaxis, cytokine and chemokine synthesis, pinocytosis, membrane phenotypes and T helper cell functions. In human DC it has been shown that the AR expression profile is related to the differentiation of cells and that Ado has distinct biological effects in immature and mature DC (136-144). Extracellular Ado induces apoptosis of immature thymocytes or even deletion of T and B cells during Ado deaminase deficiency (ADA) (142). Under hypoxia conditions, Ado can also inhibit peripheral T cell proliferation and cytokine production through A_{2A} activation. Ado inhibits the ability of activated NK cells to destroy tumor cells and this effect is rapid and induced at rather low concentrations. Both perforin- and FasL- pathways can be inhibited by Ado through cAMP-elevating A_{2A} receptors (142). However, the effect of Ado on antigen cross-priming by DC at different stages of differentiation and activation had not been earlier investigated.

The **A_{2A}** receptor, acting through a G_s protein and cAMP, has been most extensively studied and was shown to downregulate activated immune cells and to limit the in vivo inflammatory response to LPS. The accumulated extracellular adenosine at sites of inflammation has thus been suggested to serve as “reporter” molecule and A_{2A} receptors as “sensors” for the prevention of damage to surrounding normal tissues (144), although the role of other ARs is less well known. The two-danger-model (144) states that immune cells not only destroy pathogens but can also cause collateral injury to normal tissues if not tightly controlled. The surprisingly low incidence of postinflammation damage to

normal tissues indicates danger-sensing physiological mechanisms act by negative feedback inhibition. It has been suggested that a local decrease in the oxygen supply (such as occurring at sites of inflammation and in tumor tissues) results in an increased concentration of Ado which, by acting on Ado A_2 receptors and downregulates immune and inflammatory responses. Activation of high-affinity A_{2A} and low-affinity A_{2B} receptors (G_s-coupled) causes the accumulation of intracellular cAMP, which has a strong immunosuppressive effect (144).

AIMS OF THE THESIS

1. To investigate if tissue cultured mouse myeloid DC can process the exogenous OVA protein for MHC class I presentation in a vesicular pathway (VP) distinct from the classical, cytosolic MHC class I pathway.

2. If so, to characterize the VP in terms of subcellular localization, its expression during DC activation by some TLR ligands and the mechanical disruption of the cells and its dependence on the protease Cathepsin S, cytokines including type I IFN and IL-12, as well as the adaptor protein MyD88.

3. To investigate if and how, the purine nucleoside Adenosine influences the cross-presentation of OVA by DC.

4. To detect the loading of some exogenous and newly synthesized tumor-associated glycolipids onto mouse CD1d presenting molecules expressed on viable cells, including DC.

RESULTS AND DISCUSSION

Vesicular MHC-I processing pathway (papers 1-3)

Part of the present thesis work is an extension of earlier findings in our group which showed that T2-Kb/Db cells could present Sendai virus antigen in a BFA-resistant endolysomsome-like compartment (145, 146). T2 cells are defective in the expression of TAP, therefore impaired in the endogenous antigen processing for MHC-I presentation. We have here used in vitro grown DC and the model antigen OVA due to the availability of several established methods for the detection of the generated SIINFEKL peptide bound to Kb, namely mAb 25-D1 (147), T-hybridoma B3Z (148) and OT-1 transgenic mice (149). The T hybridoma assay is a more sensitive method to detect physiological levels of SIINFEKL/Kb complexes but disadvantages of this assay are the difficulties in directly quantifying the SIINFEKL/Kb complexes and the quantitative variations in the T hybridoma response. For that reason the use of flow cytometry after staining with the mAb 25-D1 represents a complementary method.

We generated DC from mouse bone marrow in vitro in the presence of rGM-CSF and rIL-4, according to the established protocol by Inaba K (25) with minor modifications (96). We named the day 5 proliferating/clustering DC immature DC (iDC), expressing an immature phenotype (low expression of MHC and co-stimulatory molecules) and sub-cultured DC resting DC (rDC), with intermediate phenotype. Activated DC (aDC) were derived from resting DC treated with stimuli, such as different TLR ligands or mechanical cellular disruption.

Paper 1

We found that iDC express a TAP-independent, BFA-, lactacystin-resistant, but lysosomotropic amine-sensitive OVA processing pathway, distinct from the classical cytosolic MHC-I processing pathway. Further, Db and Kb-binding peptides were used to follow peptide binding, internalization, exchange in Kb and transport of MHC-I/p complexes to the cell surface. We found that both iDC and rDC, and even the RMA-S (150) cell line could perform these functions, demonstrating that these events can

function independent from processing in the VP. We conclude in Paper 1 that iDC express a fully functional endolysosomal vesicular MHC-I processing pathway (VP) guided by the chaperon function of MHC-I molecules.

Activated DC (iDC and aDC) differ from rDC as that they express a highly endocytic capacity and are able to capture large amounts of extracellular fluids and cell-associated proteins by micropinocytosis and other uptake mechanisms. Also the distribution and function of lysosomes are different in activated as compared to resting DC as these in resting cells withdraw from the endocytic compartment and are tightly clustered in the perinucleus and thus show a relatively low proteolytic activity. How this is regulated remains to be established.

Peptide exchange is a well-established mechanism especially in MHC-II pathway, in which the class II-associated Li peptide (CLIP) is exchanged for a newly generated MHC-II binding peptide in the endocytic compartment by the help of H-2DM, a MHC-II-like molecule (151). Peptide exchange can also occur with MHC-I both in the ER and the endolysomsomes (151). MHC-I assembly and the formation of MHC-I/p complexes (in the case of endogenous antigens) are accomplished in the ER, by the competition with a TAP-derived endogenous peptide (152). Peptide exchange in the endosomes or in the post-Golgi compartment is important for the VP by providing this pathway with peptide receptive MHC-I (153). Peptide exchange reactions are facilitated in the acid environment of the endocytic pathway where the pH ranges from pH6.5 to pH5 or even lower in lysosomes (154). The optimal pH for peptide exchange is usually in the range of pH4.5-5.5 (155).

In our peptide exchange system, we first blocked the routes for the nascent MHC-I molecules using BFA which disrupts the Golgi function. Then we loaded a stabilizing peptide at 37°C to saturate surface and endosomal MHC-I by peptide exchange and also to allow the internalization of free peptides. After washing away the free unbound peptides in the presence of BFA, the second peptide was added at 37°C or 15°C (non-internalizing conditions) to look for the potential peptide exchange intracellularly or at

the cell surface. Results showed that peptide exchange in MHC-I exist at 37°C, but not at 15°C, suggesting that it only occurred in the intracellular compartment as peptide binding to membrane MHC-1 readily occurs at 15°C (data not shown).

Paper 2

We further explored the VP by using a T cell hybridoma (B3Z) recognizing the same structure as the 25D-1 mAb does. We reproduced the findings in paper 1 by showing that day 5 iDC transiently expressed an endolysosomal vesicular MHC-I processing pathway. In addition, we also found that this VP could be restored by CpG ODN activation of sub-cultured rDC. Both iDC and CpG-activated DC could regurgitate antigenic materials to the cell surface for the binding to surface MHC-I molecules, consistent with earlier findings that activated macrophages can operate a TAP-independent MHC-I processing by peptide regurgitation (87). We also show in paper 2 that iDC secret soluble proteases which can process OVA at the extracellular phase. We conclude in this paper that iDC express multiple pathways for the processing of OVA for MHC-I presentation.

A special non-cytosolic compartment for the processing of extracellular antigens for MHC-I presentation has been described by other groups using different forms of antigens, including bacterially fused OVA (87-90), bead-coupled OVA (91, 92) cell-associated viral particles (93-95) as well as soluble OVA as used in paper 1 and this paper. Phagocytes have two compartments for proteolysis, endolysosomes and cytosol. Endolysosomal MHC-I processing may produce quantitatively and qualitatively different peptides as compared to those formed in the classical, cytosolic pathway. It would thus be interesting to isolate MHC-I bound peptides from these two different processing compartments to establish the amount of over-lapping. Over-lapping peptides could be formed due to the presence of carboxypeptidases in endolysosomal compartment and the similar MHC-I chaperon function in both compartments. However, an immunogenic peptide formed only in endolysosomes after the internalization of a secreted tumor antigen has been recently demonstrated in a tumor system (109, 156). CTL with specificity for such peptides could thus kill target cells which can present them, like

certain types of tumors and possibly also DC and other phagocytic cells as part of an immunoregulatory function.

The endocytic compartment contains cysteine proteases and other enzymes. A major breakthrough for the endocytic MHC-I processing is the finding that the VP requires the cysteine protease Cathepsin S (Cat S). OVA incubated with isolated phagosomes from wt DC resulted in the formation of SIINFEKL/Kb complexes but this did not occur with phagosomes isolated from the Cat S knockout DC. Moreover purified Cat S itself can process OVA into the SIINFEKL peptide. In one study, it was also found that the Cat S knockout mice mounted impaired CTL responses against two viral epitopes, demonstrating a critical role of this protease also in vivo (91).

Where does the SIINFEKL peptide bind to Kb after its generation in the VP? In the endolysosomes or at the cell surface after regurgitation? Regurgitation of peptides to the cell surface was demonstrated in some system using bacterially fused OVA and activated macrophages (87) and also in our own system, but was not always found reproducible (91), suggesting the possibility of intra-vesicular peptide binding. Most groups used fixed (paraformaldehyde) APC as the responder cells for the assessment of regurgitation. Fixation provides a high level of empty MHC-I molecules, by chemically cross-linking, a procedure which may not be representative for in vivo conditions. If the formation of MHC-I/p complexes happens within the VP, the next question would be how MHC-I molecules traffic to this compartment? Internalization by invagination from the plasma membrane could bring MHC-I molecules to the endosomal compartment. Supporting this possibility is that the VP was operated in the presence of BFA, which blocks transport of nascent MHC-I proteins from the ER, although long-term (6h) incubation with BFA may deplete MHC-I molecules in the post-Golgi pool. As an alternative route, MHC-I might be targeted into endosomes by the Li chain directly from the ER (106, 157) and lastly membrane MHC-I molecules could constitutively recycle between the cell membrane and the endosomes (105).

Of particular interest in paper 2 is the finding that an immunostimulatory CpG ODN could restore the vesicular MHC-I processing pathway (VP) in subcultured resting DC. CpG, a TLR-9 ligand has been shown important for cross-priming (158, 159) and also inducing the early upregulation of endocytosis (160). CpG has been used as an adjuvant in both tumor immunotherapy and also for the control of models of autoimmunity (8, 161). DC activation by CpG may be accompanied by an alteration of the endocytic compartment and the trafficking of MHC-I molecules, in addition to the upregulation of the endocytosis. We thus considered it interesting to investigate if CpG is unique with regard to the restoration of the VP or if also other TLR ligands could have the same effect.

Three steps are involved for the generation of MHC-I ligands, namely degradation by the proteasomal complexes, trimming in the downsteam of proteasomes and in the ER and the rapid destructions of the peptides if these are not chaperoned by MHC-I molecules. It has been estimated that non-proteasomal processing could contribute to one third of the all MHC-I bound peptides (162). Our finding that iDC can secrete proteases into the supernatant, which process OVA for presentation on H2-Kb, is similar to that described in a MHC class II processing system (163) using HEL and beta-casein proteins, Santanbrogio et al. reported that supernatants from iDC process these proteins for MHC class II presentation as they contained molecules required for peptide loading onto MHC class II at the cell membrane (163). The processing by secreted proteases in our system is impressive, as the OVA protein has a quite complicated structure containing three domains, subdomains and a high number of disulfide bonds. The position of the SIINFEKL peptide (aa 257-264) in domain II and the flanking sequences may confer a special sensitivity to the involved proteases, explaining the immunodominant character of this CTL epitope (164). At present we have no clear idea which proteases are involved during extracellular processing, although preliminary experiments with inhibitors (data not shown) implicate serine proteases (inhibition by AEBSF) but not aspartic amino acid proteases (no inhibition by pepstatin). Interestingly, the cysteine protease inhibitor E64 increased OVA processing markedly, demonstrating that the supernatants probably contain a mixture of proteases which can both favor and inhibit formation of the

particular H2-Kb binding SIINFEKL epitope in the present system. As supernatant processing is quite slow (requiring 48 h), it probably reflects only part of a more efficient in vivo processing system that includes both secreted and endolysosomal proteases. This supposition is supported by the faster and more efficient processing detected in the regurgitation assay (92). Nevertheless, our results with secreted proteases demonstrate a remarkable redundancy in MHC class I processing pathways and should be helpful in the further characterization of proteases involved in this type of processing.

Paper 3

We further characterize the VP by using Cathepsin S (Cat S) knockout mice and also by investigating other stimuli in terms of the capacity to restore VP in resting DC, namely LPS (TLR-4), Zymosan (TLR-2) and mechanical disruption. The vesicular MHC-I processing of OVA could be directly visualized in wt DC but not in the Cat S-/- cells and the latter cells also failed to process OVA in the VP by iDC and CpG-activated DC in the present system, consistent with the early findings by Shen et al (91). LPS and disruption can both active the VP, but not zymosan. We further show that the functioning of this pathway does not depend on type 1 interferon and IL-12, but requires the adaptor protein MyD88.

Different TLRs, including TLR-2, 4, 3 and 9, have earlier been found to be important for the cross-presentation of exogenous antigens for MHC-I restricted T cell responses (8, 161) and this is also true for the TLR-linked MyD88 adaptor protein in several independent in vivo systems (165, 166). However, which MHC-I processing pathways that DC employ to generate these in vivo responses is presently not clear although the in vivo importance of the VP has been clearly demonstrated in other model systems (91, 110). DC activation through TLR receptor stimulation can lead to both the production of different inflammatory cytokines and type I IFN (2, 161). However, neither type I IFN nor the IL-12 cytokine (at least in the case of VP activation by cellular disruption) seems to be involved in the activation of the VP in the present system. Thus, other inflammatory cytokines might be involved.

Interestingly, a mere mechanical disruption of cells by repeated pipetting could also activate the VP in resting DC as earlier demonstrated by Delamarre et al. in a similar system (167). The disruption-induced VP was detectable at 4h, peaked at 24h and was further increased at this time point by the simultaneous CpG stimulation. VP activation by cellular disruption was found to be independent of IL-12 synthesis in the cultures and the development of an activated cellular phenotype. Cellular disruption of DC clusters has earlier been shown to induce the maturation of cells and to potently stimulate cross-presentation by DC (167). Although cellular disruption can lead to the release of heat shock proteins (168), an event which might influence the endosomal compartment without altering the cellular phenotype of cells, the underlying MyD88 dependent mechanisms that explain VP activation by cellular disruption remains unclear. Mechanically stressed cells may use "mechanotransduction" for the activation of transcription factor and genes coding for, for instance, inflammatory cytokines (169). This can be further studied using expression arrays and proteomics.

Zymosan did not induce VP expression in resting DC but rather favoured cytosolic processing. Zymosan is a substance derived from the yeast cell wall and composed of polysaccharide chains of various molecular weights. It directly activates macrophages and induces strong inflammatory responses but it is not degradable (170). This property might facilitate the transport of OVA from the uptake vesicle to the cytosol by "leakage" and thus favours the expression of the classical cytosolic MHC-I pathway.

Vesicular processing for MHC-I presentation is similar to the classical MHC class II processing and presentation pathway in depending on defined cellular events such as uptake of exogenous antigens, the intra-vesicular processing of these, loading of peptides onto MHC molecules and the transportation of these complexes to the cell surface (171). Which of these steps that are activated in resting DC to allow the expression of the VP and how this occurs in response to the different stimuli in the present system are unclear. There are several possibilities like the regulation of Cat S activity by the cystatin C (anti-protease) (54, 172, 173) or the regulation of the vesicular trafficking, both to the processing compartment and to the cell surface (174). The presently generated knowledge

in terms of the expression of vesicular and cytosolic MHC-I processing pathways at different stages of DC maturation and activation could be clinically useful if extended to human cells. For instance, both active vaccination and cellular DC immunotherapy against cancer and infectious diseases might be optimized if multiple MHC-I processing pathways are exploited in a controlled way.

DC have many functions, including the induction of self-tolerance, maintenance of the peripheral naïve T cell pool, induction of immunity against infectious diseases as well as the induction of regulatory T cells which can suppress the excessive immune responses and prevent autoimmune diseases. DC originate from hematopoietic progenitors in the bone marrow which seed into the spleen, the lymph nodes and other lymphoid tissues via the blood. "Blood-derived" DC constitute 100% in the spleen but 50% in LN as LN can also receive DC from the afferent lymph vessels (Figure 17). Monocytes in the blood can reside in tissues and further develop to iDC in mucosal, dermal or epidermal sites, as the Langerhans cells (LC).

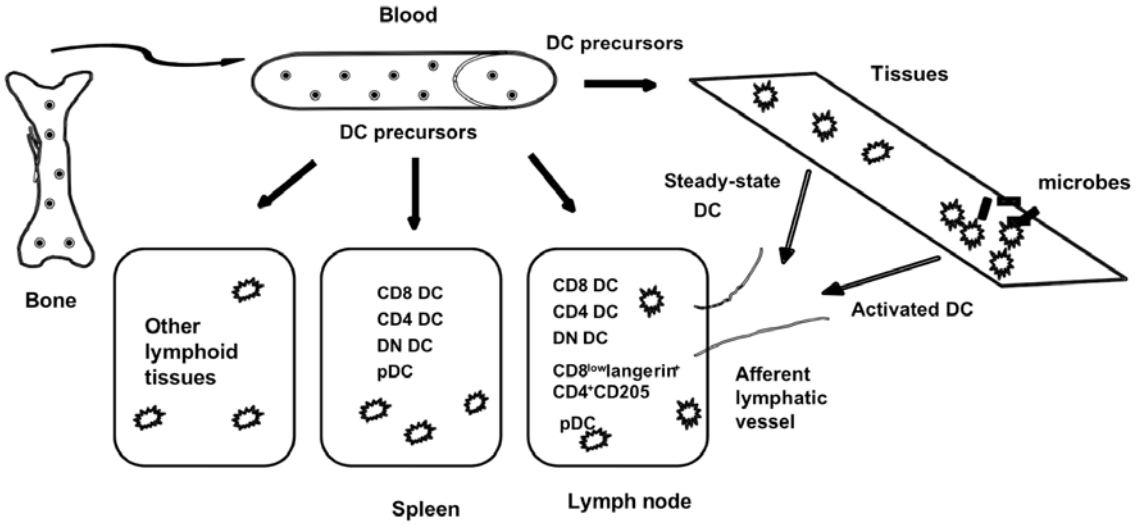

Figure 17 DC development in vivo.

Our in vitro DC system may be somewhat representative for the DC development in vivo with proliferating immature DC (our day 5 iDC) present in lymphoid tissues and rDC and aDC in peripheral tissues, representing steady-state and microbially activated cells,

respectively (Figure 17). Steady-state resting DC (our rDC) may be important for the sampling of tissue-derived antigens and the presentation of these in lymphoid tissues to maintain the peripheral self-tolerance and the size of the peripheral naive T cell pool. Immature DC in lymphoid tissues (our iDC) may have similar functions in relation to internalized, exogenous self-antigens. Upon microbial infections (our aDC) these DC migrate to the draining LN for the presentation of the antigens to responsive T cells. The outcome of this interaction in terms of tolerance-induction, T cell priming or even regulatory T cell expansion differs according to the context of the activation step. Cytokines (signal 3) together with the co-stimulatory molecules (signal 2) and TCR-MHC/p interaction (signal 1) cooperate and determine the end function of DC.

Day 5 DC (iDC) in our culturing system may thus represent cells that are found in the lymphoid tissues entering from the blood. It has been shown that the turnover of DC is fast (2-5 days) requiring a considerable constant input of DC precursors to maintain the DC homeostasis. In addition, in contrast to the in vivo situation our iDC are totally CD8 negative which may be explained by differences in vivo and in vitro in terms of amounts and composition of the local cytokines during the formation of DC.

The expression of the VP at different stages of DC maturation and activation might thus allow the processing and presentation of both self and microbial antigens for the fulfilment of several different DC functions,

Cross-presentation regulation by Ado (Paper 4)

Levels of the purine nucleoside adenosine (Ado) increase during hypoxic conditions such as inflammation, tumor growth and tissue damage. The amount of the increased extra-cellular adenosine can range from 10 to 20 μM, with its physiological level being only 20-300 nM (132, 134). Ado can exert a large variety of effects on many different tissues and organ system, including the immune system. For the past years, Ado has been shown to regulate some parameters in DC biology, including phenotypes, cytokine and chemokine secretion through one or more of the four known Ado receptors that are differently expressed in resting and activated cells. However, to our knowledge, there

were no earlier studies on the effect of Ado on the cross-presentation function in DC. This might be important to study as DC often are present and functional at sites which typically can contain elevated levels of Ado.

In this paper we investigated how cross-presentation of exogenous OVA was affected by Ado. We used a stable form of Ado, 2-chloro-Ado and the already established cross-presentation system, used in paper1-3 for this study. We found that cross-presentation was strongly and dose-dependently inhibited by Ado in resting DC but did not have this effect in iDC and aDC. This inhibition could be overcome by an Ado receptor antagonist (CGS15943) and by pertussis toxin, a G_i-protein antagonist, demonstrating that it was mediated by an Ado receptor (A_1 or A_3). By quantitative PCR, we found that the expression of the A_1 receptor mRNA was increased in resting DC and, in addition, that the inhibition was not seen in resting DC derived from A_1-/- mice. We concluded the inhibitory effect in the present system was mediated by the A_1 receptor.

We next asked which step in the cross-presentation process was inhibited by Ado. In paper 1-3 we suggested the rDC processed exogenous OVA by the "leakage pathway" in which internalized OVA somehow entered the cytosol and merged with the classical MHC-I processing pathway. In order to know if Ado blocked classical MHC-I processing we intentionally introduced OVA into the cytosol by electroporation in the presence of Ado, BFA and lactacystin, the latter two being the Golgi and proteasome inhibitor, respectively and measured the formed SIINFEKL/Kb complexes on the cell surface by flow cytometry. We found that Ado had no inhibitory effect on the processing of cytosolic OVA in contrast to BFA or lactacystin which inhibited this process. In addition, Ado did not inhibit the uptake of FITC-OVA into cells or the binding of exogenous peptide to Kb. Intracellular staining showed that the SIINFEKL/Kb complexes were retained intracellularly in the presence of Ado. We conclude from this study that adenosine suppresses cross-presentation in resting DC by acting on the A_1 receptor and this inhibition is a consequence of the retention of intracellular MHC-I/p complexes.

This part of the study seems contradictory to our earlier findings (papers 1-3) that resting DC mainly process OVA through the classical cytosolic MHC-I pathway, compared to iDC and aDC using a vesicular MHC-I processing pathway (VP). The question is why BFA blocked OVA processing in resting DC, not iDC and aDC. Can BFA have other functions than blocking the Golgi apparatus? BFA is a fungal metabolite that has profound and dramatic effects on the secretory pathway. It not only inhibits secretion, but also causes massive morphological changes causing by the rapid loss of β-COP, the latter is usually found throughout the Golgi stack. Typically BFA induces the Golgi apparatus to disintegrate so that many Golgi enzymes are redistributed to the ER. However, these changes are fully reversible when the drug is removed (175). Of particular interest is that BFA also has an effect on the endosomal system. β-COP is not found in endosomes (175), instead the target for BFA here is the sec 7 domain (176, 177) which is present in guanine Arf nucleotide-exchange factors (Arf-GEFs) involved in the recruitment of coat proteins important for vesicular formation and trafficking both in the Golgi and in the endosomal systems (176, 177). It is thus possible that endosomal recycling in resting and activated DC is differently regulated in such way that the process becomes resistant to BFA in cells expressing an activated phenotype, possible by becoming independent of Arf-GEFs or on Arf-GEFs with a sec7 domain insensitive to the inhibitory effect of BFA such as CYT and EFA6 (176, 177). If so, the level of DC activation has to be considered when BFA alone is used to characterize MHC-I processing pathways.

A_1 receptor signalling can influence membrane dynamics through effects on actin re-organization. The cytoskeleton regulates many events including cell morphology, motility and possibly endosomal activities. Thus it is possible that Ado acting on the A_1 receptor in rDC inhibits a VP present in these cells which is qualitatively and possibly quantitatively different from a VP expressed in activated cells (iDC and aDC). If so, as mentioned above, this has a bearing on the interpretation of the VP expression in rDC in papers 1-3 although it also might further indicate the wide expression and significance of the VP.

Loading CD1 with glycolipids in living cells (Paper 5)

In this paper we shifted to exogenous glycolipid presentation by mouse CD1d molecules. CD1 molecules are MHC-I-like but non-polymorphic antigen presenting molecules often presenting lipids or glycolipids for NKT cell recognition. These can rapidly respond (2 h after antigen administration) producing large amount of cytokines, including IFN-γ and IL-4, and have profound anti-tumor and immuno-regulatory effects. Administration of the well-known CD1d-binding ligand, αGalCer, a marine derivative (178), has been shown to cause the regress of metastatic tumor disease in some systems (179, 180). There are five CD1 variants in humans and one in mice (CD1d) in mice. CD1 molecules are surface glycoproteins structurally similar to MHC-I molecules, in that they consist of a heavy chain non-covalently associated with a β_2-m chain but different as they function independent of TAP, are non-polymorphic, traffic through endosomes and show a restricted tissue distribution.

CD1 presentation of certain glycolipids, which carry tumor associated carbohydrate antigens (TACAs) may trigger anti-tumor effects. Due to the difficulties involved in vaccination with hydrophobic molecules like glycolipids we reasoned that such antigens might be loaded onto DC in vitro for the possible use in cellular immunotherapy against cancer. As most studies on glycolipid binding to CD1 had earlier been done using cellular assays or solid-phase bound CD1 we decided to develop a method to quantitatively estimate the binding of glycolipid ligands to CD1 expressed on living cells.

Using four newly synthesized glycolipds, bearing TACAs, we loaded these onto mouse CD1d molecules in living cells, including DC and EL4 cells, a lymphoma cell line. The binding assay was based on using mAb which specifically recognized the carbohydrate part of the glycolipid structures. We found that such TACAs could be detected by flow cytometry on wt EL4 cells but not on β_2-m knockout EL4 cells (lacking CD1d expression) suggesting the loading was specific for CD1d molecules. To further establish the selectivity of the binding, we used the well-known CD1d ligand αGalCer and found that loading was inhibited by the addition of aGalCer in a dose-dependent manner, indicating the glycolipid binds to CD1d in a way similar to αGalCer. Lastly we found

that the loading was completely abolished in CD1d knockout cells. We thus successfully loaded, in a quantitative way, our newly synthesized glycolipids, bearing TACAs onto CD1d expressed living cells, including normal DC.

Kronenberg and colleagues have shown that the orientation of the sugar is more important for TCR recognition of the glycolipid than for the CD1d contact as CD1d primarily binds to the hydrophobic acyl chains (56). With that in mind, our newly synthesized glycolipids can be tested for immunogenicity (or other effects) in mice challenged with tumors expressing the corresponding TACAs. In addition, the possibility for cross-talk between the innate and adaptive immune systems and activation of one of the systems by MHC- or CD1-restricted T cell recognition is poorly understood. Interestingly, it was earlier found that immunization of mice with a class I MHC-binding peptide carrying a galabiose structure in a central position allowing TCR recognition generated a subpopulation of T cells which could lyse cells loaded with a galabiose containing glycolipid in a MHC-unrestricted manner (181, 182). A strategy based on immunizations with tumor-associated carbohydrate antigens bound to different carrier molecules, such as MHC-binding peptides, CD1-binding lipids, or proteins activating helper T cells, might thus allow the selective activation of different effector mechanisms in the immune system with distinct anti-tumor effects.

CONCLUSIONS AND FUTURE PERSPECTIVES

1. We have clearly identified a highly efficient vesicular MHC-I processing pathway (VP) expressed in activated in vitro cultured DC (iDC and aDC) which is distinct from the classical cytosolic MHC-I pathway.

It is of obvious importance to identify this VP in DC isolated from in vivo, at different stages of differentiation and activation.

Another important aspect is to further characterize the VP at the cellular and molecular levels in terms of the intracellular processing compartment, the involved proteases and mechanisms that direct vesicular trafficking in activated as compared to resting DC. These studies can be done with cellular, biochemical and genetical (gene expression in microarrays) methods.

Another future direction would be to perform functional studies which aim at defining the biological importance of the VP in terms of generating immune responses and other DC functions such as the generation of tolerance to soluble self-antigens and the maintenance of the peripheral naïve T cell pool. To this end the present system can easily be extended to in vivo conditions by the use of the transgenic OT-1 mice strain which express the same TCR as our B3Z hybridoma, recognizing the SIINFEKL/Kb complex.

2. We have demonstrated that the purine nucleoside Ado, through the A_1 receptor, suppresses cross-presentation in resting DC, most probably by retaining MHC-I/peptide complexes in a BFA sensitive VP expressed in these cells.

The VP in resting DC may be involved in the presentation of soluble self antigens in secondary lymphoid tissues. The outcome of such a presentation is presently not clear but could be studied in mice lacking the various Ado receptors. Genes for all four receptors

(A_1, A_{2A}, A_{2B} and A_3) have now been silenced (knockout mice) and will be available at the KI (Bertil Fredholm's group). A finer characterization of possible immune aberrations (altered immune responses or tolerance mechanisms) in such mice might indicate biological functions for the respective receptors. Already, A_{2A} KO mice have been found to be less capable of controlling inflammatory responses as compared to wt mice.

We have also found that different Ado-R KO mice differ dramatically in response to a subcutaneously injected tumor (RMA lymphoma) (data not shown) and these studies need to be extended to include possible mechanisms such as the trafficking of inflammatory cells to the tumor site and the local immune functions that these perform there.

There is now also a respectable number of Ado-R agonists and antagonists produced by the pharmacological industry. Possibly some of these could be used for therapeutic immune interventions.

3. We have been able to visualize and quantitate the loading of newly synthesized glycolipids (GLs) containing TACAs onto CD1d molecules expressed on the surface of living cells, including EL4 and DC cells.

It will be interesting to immunize mice with DC loaded CD1d molecules with glycolipids containing TACAs and investigate the effect on tumors expressing the corresponding TACAs. By using differently loaded DC, combined with or without specific adjuvants, different NKT cell responses might be generated which either promote or prevent tumor growth. In the long-term, these types of studies might eventually define procedures which generate clear anti-tumor effects similar to what has been seen with DC loaded with tumor-derived MHC-I binding peptides.

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