Processing of MHC class II in dendritic cells

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Processing van MHC klasse II in dendritische cellen
(met een samenvatting in het Nederlands)

Proefschrift

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Promotor: Prof. Dr. W. Stoorvogel

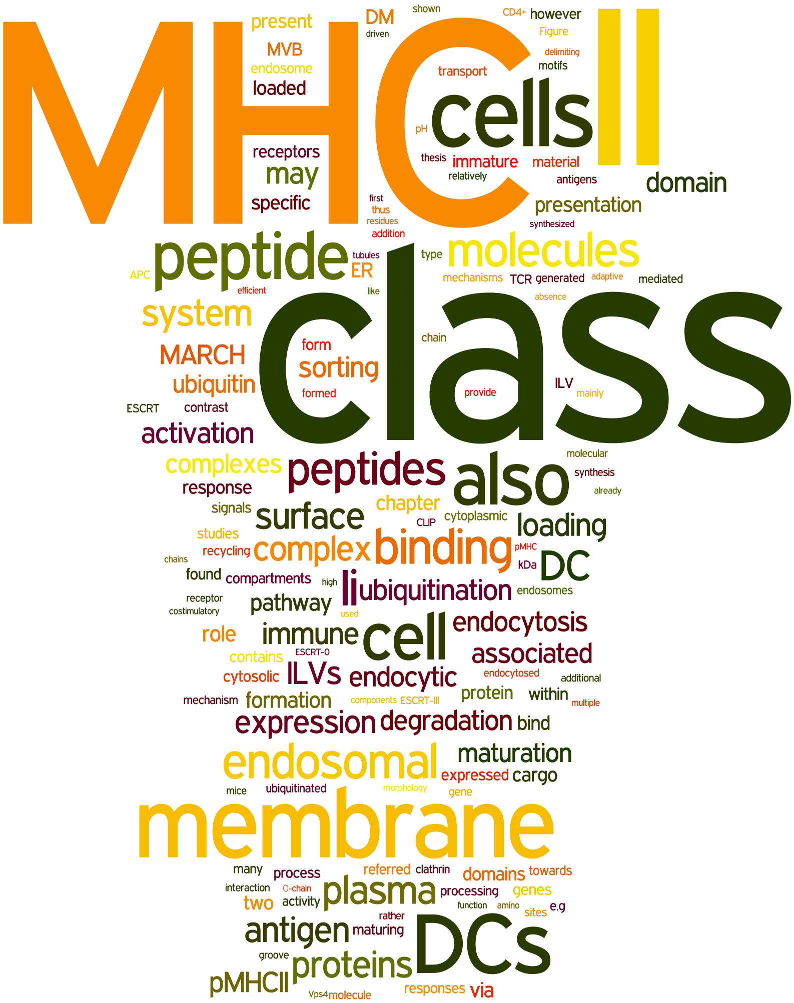
Co-promotor: Dr. R. Wubbolts

Table of contents

Abbreviations

APC	antigen presenting cell
AMSH	associated molecule with SH3 domain of STAM
BMDC	bone marrow derived DC
CHMP	charged multivesicular body protein
CLIP	class II associated invariant chain peptide
DC	dendritic cell
DUB	deubiquitinating enzyme
EAP30	ELL-associated protein of 30 kDa
EE	early endosome
ER	endoplasmatic reticulum
ERAP	ER aminopeptidase
ESCRT	endosomal complex required for transport
GILT	γ-interferon induced lysosomal thio reductase
GLUE	GRAM like ubiquitin-binding in EAP45
HECT	homologous to E6 carboxy terminus
HLA	human leukocyte antigen
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IL	interleukin
ILV	Intraluminal vesicle
Ii	invariant chain
LPS	lipopolysaccharide
IS	immunological synapse
LAMP1	lysosome associated membrane protein 1
LE	late endosome
LFA-1	lymphocyte function-associated antigen 1
LIP5	lyst-interacting protein5
MARCH	membrane-associated RING-CH
MHC	major histocompatibility complex
MIIC	MHC class II compartment
MIM	MIT-interacting motif
MIT	microtubule-interacting and trafficking
MVB	multi-vesicular body
PI	phosphoinositide

pMHCII	peptide loaded major histocompatibility complex II
PRR	pattern recognition receptor
RE	recycling endosome
RING	really interesting new gene
SE	sorting endosome
SMAC	supra-molecular activation complex
STAM	signal transducer adaptor molecule
TAP	transporter associated with antigen presentation
TEM	tetraspanin-enriched microdomains
TCR	T cell receptor
TGN	trans-Golgi network
TLR	toll-like receptor
TNFα	tumor necrosis factor α
Tsg101	tumor susceptibility gene 101
UBD	ubiquitin binding domain
UBPY	ubiquitin peptidase Y
UEV	N-terminal Ubiquitin E2 Variant
UIM	ubiquitin-interaction motif
VPS4	vacuolar protein sorting 4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General introduction

General introduction

Dendritic cell Biology

Already in 1868, Paul Langerhans described a cell type in human skin which displayed a nerve cell-like morphology. Based on their morphological characteristics, he mistakenly believed that these Langerhans cells were part of the nervous system. In 1973, Steinman and Cohn identified a cell with a similar morphological appearance in lymphoid organs [1]. Because of their branched projections, or dendrites (from the Greek δένδρον = tree), these cells were termed dendritic cells (DCs). Although an immunological role of Langerhans cells had emerged in the meantime it was not until 1985 that it became evident that also they belonged to the DC category [2]. DCs turned out to be potent stimulators of T cell proliferation [3] and are currently considered to represent the most powerful antigen presenting cell (APC) in the immune system.

The immune system of vertebrate organisms provides protection against pathogens and operates through a two-level defense mechanism. The first is the innate immune system, which consists of barriers created by skin or mucus and the immediate and constitutive action of macrophages, granulocytes, mast cells, dendritic cells, natural killer cells and the complement system. Clearance of pathogens by the innate immune system is rather unspecific and initiated by means of receptors on cells or elements of the complement system that bind molecular patterns that are commonly expressed by pathogens but absent on host material. The second layer of defense is the adaptive immune system, which neutralizes pathogens through the generation of antigen-specific responses. Adaptive immunity involves both the generation of antigen-specific antibodies, referred to as the humoral response, and the development of cells that specifically kill diseased or infected cells. These responses are highly specific for the invading pathogen but require 4-6 days from the time that antigen is encountered to become effective [4]. The acquired resistance against a specific pathogen after a first infection was observed already centuries ago. With the introduction of genetics it was soon determined that the relevant genes

mapped to the major histocompatibility complex (MHC) [5]. In humans, the MHC is the most gene-dense region of the genome and it contains at least 200 genes of which more than 20% is functional in immunity. The central role of MHC molecules is for instance reflected by many autoimmune diseases, e.g. diabetes and rheumatoid arthritis, which are genetically linked to these genes.

Besides playing their part in the innate immune system as a phagocyte, DCs plays a vital role in the initiation of adaptive immunity. DC-precursors are generated in the bone marrow, and via the blood circulation enter the peripheral tissues where they attain DC-specific characteristics. In this "resting" or "immature" state they continuously sample their environment by taking up material using multiple endocytic mechanisms. In this way, a "library" of endogenous "self" material present in the vicinity of the DC gains access to the DC interior, which is used to develop and maintain peripheral tolerance against this "self" material. Internalized proteins are unfolded and processed into small peptides that may associate with MHC class I or class II molecules. These MHC-peptide complexes can then be transported to the cell surface for inspection by other immune cells [4, 6]. It has become evident that in vivo, DCs represent different classes of specialized cells [7, 8]. DCs can be classified in two main categories, the conventional DC (cDC) and the plasmacytoid DC (pDC) [9, 10], each of which can be subdivided into several subtypes [11]. These DC subtypes are characterized by the presence or absence of specific surface markers, their location in the body and functional features. Specialized functions of DC subtypes are exemplified by the efficient anti viral response by pDCs or the potent MHC class I cross-presentation by $CD8^+$ DCs, a process which will be discussed later in this chapter.

Maturation of the dendritic cell

In the absence of inflammatory cues, DCs dynamically populate peripheral tissues. During this "sentinel" or "immature" state they survey their environment with an array of receptors which can recognize conserved pathogen associated molecular patterns (PAMPs). Stimulation of these innate pattern recognition receptors (PRRs) initiates a differentiation process which is termed maturation

and involves many changes in DC function [12, 13]. PRRs include Toll-like receptors (TLRs) [14], C-type lectin receptors [15], RIG-I-like receptors and Nod-like receptors [16]. DCs also express receptors for inflammatory cytokines which can function in an autocrine or paracrine fashion to trigger DC maturation. Together these exogenous and endogenous "danger" signals induce the necessary transformations to equip DCs for priming adaptive immune responses. Characteristic features of DC maturation include an increased surface expression of costimulatory molecules (e.g. CD86, CD80, CD40) and facilitated migration from peripheral tissues to the local lymphoid tissues where they may interact with other immune cells (e.g. T cells) (Figure 1). Although the phenomenon of DC maturation was first described as a number of phenotypic changes needed for DC mediated immunity, it must be noted that similar changes have also been seen in DCs that induce tolerance rather than immunity [17, 18].

Numerous genomics studies have shown a profound reorganization of gene expression within the first hours of DC maturation [19.] Changes in gene expression are determined by the type of DC stimulation. For instance, DC activation by lipopolysaccharide (LPS), an inflammatory microbial constituent, or Tumor Necrosis Factor (TNFα), a nonmicrobial endogenously produced stimulus, resulted in distinct gene expression profiles [20]. LPS exposed DCs rapidly display a potent mature phenotype that is optimal for eliciting an immunogenic response. In contrast, TNFα stimulation results in a "semi-mature" state which is tolerogenic rather than immunogenic due to the ability of these DCs to induce IL-10 producing T cells [21-23]. This and other examples indicate that the nature of the encountered stimulus determines the type of DC maturation, illustrating the high plasticity of DCs during their development [19].

DC maturation is characterized by a dramatic subcellular reorganization, most obviously reflected by the formation of many dendrite-like extensions of the plasma membrane, surface membrane folds and cytoskeletal rearrangements. In response to activation signals, DCs immediately increase their endocytic capacity, thus facilitating enhanced antigen capture [24-27], but also upregulate MHC class II synthesis [20, 28-33]. Both endocytosis and MHC class II synthesis are only transiently upregulated and greatly diminished in fully matured DCs. In addition, MHC class II is redistributed within the cell from a characteristic late endosomal compartment in immature DCs to the plasma

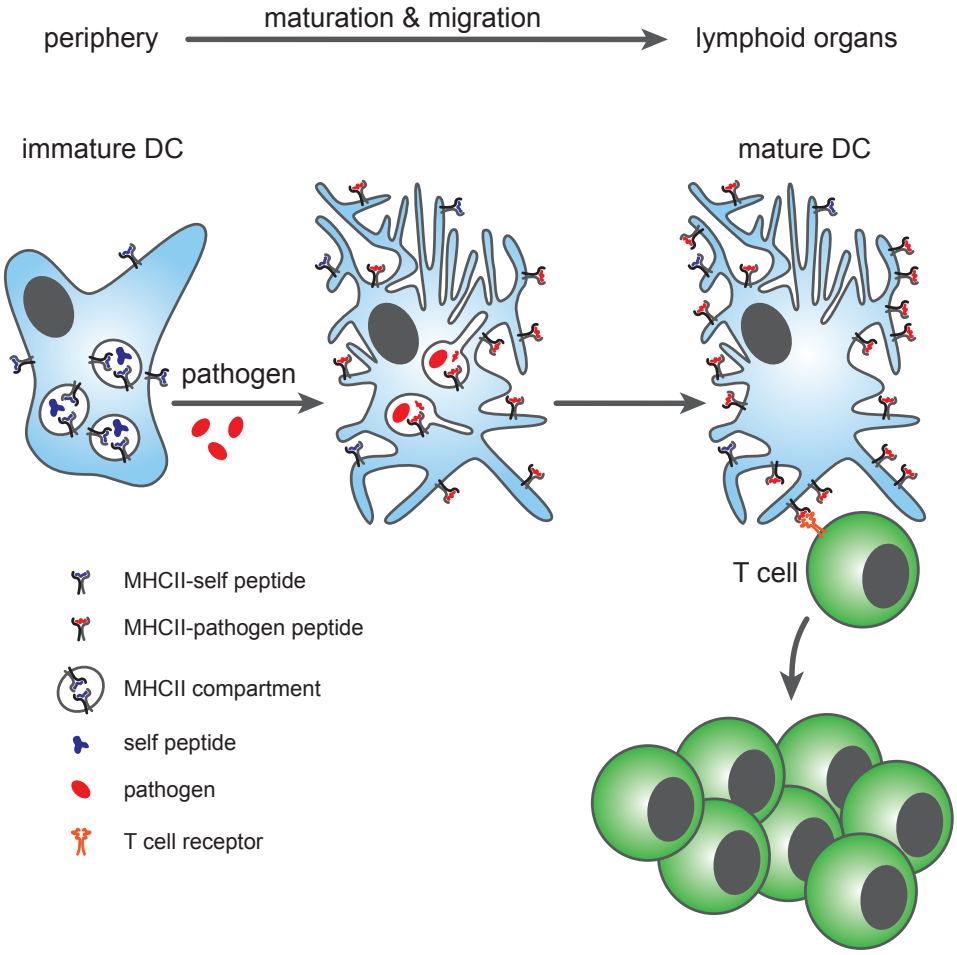


Figure 1. Pathogen derived peptides are presented by MHCII on DCs
Immature DCs in peripheral tissues load their MHCII molecules in endosomes with self peptides. When DCs encounter a pathogen they are triggered to initiate a maturation program. During this process, the pathogens are endocytosed and processed into peptides, which can be loaded onto locally present MHCII. Maturing DCs migrate to secondary lymphoid tissues, where they gain a dendritic morphology, and stably express newly formed MHCII peptide complexes at their plasma membrane for evaluation by T cells. For simplicity, costimulatory molecules, adhesion molecules and coreceptors are not drawn in the figure.

membrane in mature DCs. During DC maturation, an endosomal tubular network is formed that functions in the transfer of MHC class II from endosomes to the plasma membrane [34-39]. DC activation also profoundly increases the half-life of peptide loaded MHC molecules, which will be discussed in more detail later in this chapter. Finally, DC maturation is characterized by upregulated expression of costimulatory membrane proteins and cytokines, which help productive interactions of MHC molecules with target T cells.

MHC class I and MHC class II

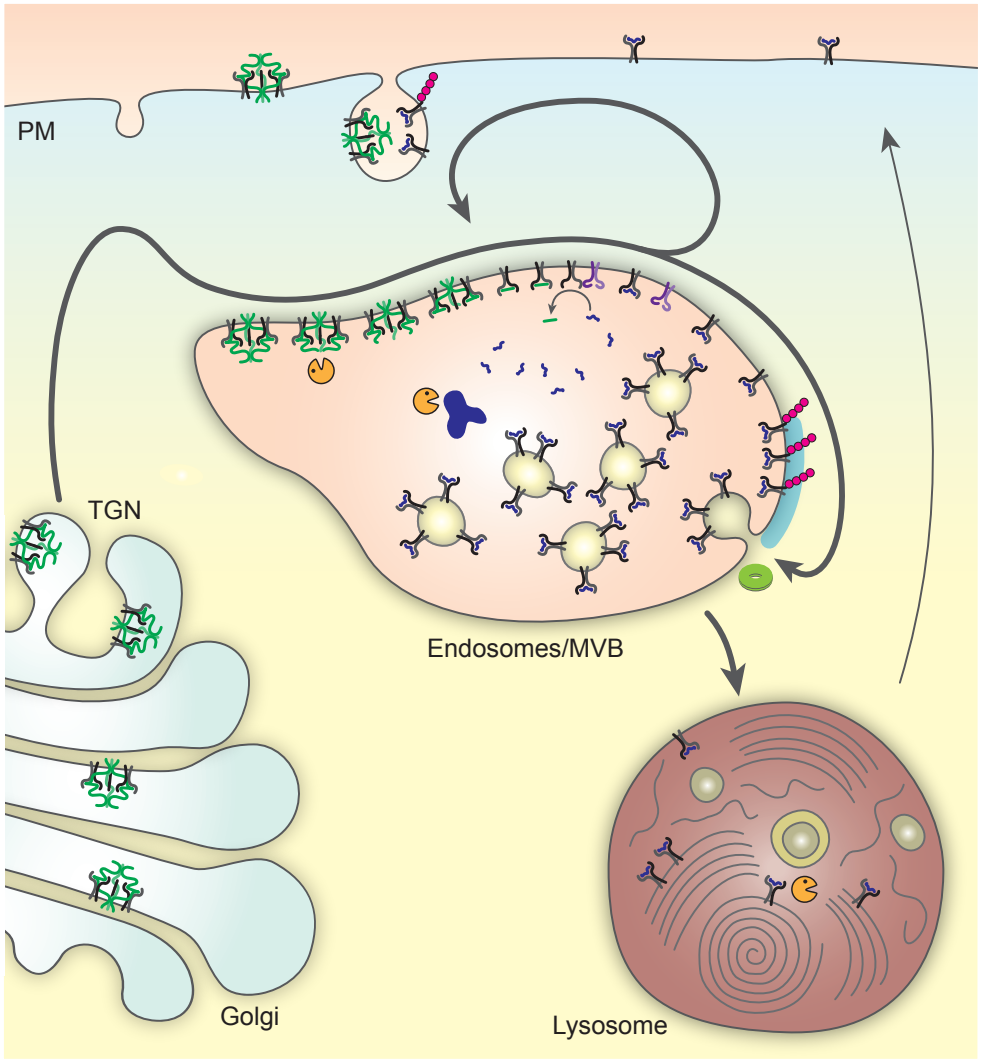
Pathogens manifest themselves in extracellular body fluids or within host cells. To control both of these potential infection sites, two types of MHC molecules have evolved, MHC class I and MHC class II. While MHC class I is expressed by all cells, MHC class II is mainly expressed by the "professional" APC to which DCs but also macrophages and B cells belong [4, 6, 40]. The MHC class I pathway predominantly helps to eliminate infected cells through the activation of antigen specific cytotoxic T cells while MHC class II primarily serves to mount humeral immune responses and to instruct regulatory or memory T cells. MHC class I and class II signaling pathways are however interdependent, particularly for the initiation of cell mediated adaptive immune responses. In non-professional antigen presenting cells, MHC class I is exclusively loaded with peptides that are generated in the cytosol by the ubiquitin/proteasome system. These cytosolic peptides are translocated into the ER lumen by the TAP (transporter associated with antigen presentation) complex where, if necessary, ER aminopetidases (ERAP) can reduce the peptide length [41]. Peptides are then loaded onto MHC class I, aided by the MHC class I loading complex [42]. Peptide association allows final folding of MHC class I and transport out of the ER via the Golgi apparatus to the cell surface, where it is stably expressed. Cytotoxic T cells recognize infected cells by their exposure of MHC class I-bound pathogen specific peptides. In contrast to other cells, DCs have an additional capacity to load exogenously acquired peptides onto MHC class I. This process is referred to as cross-presentation and will be discussed in more detail later in this chapter.

MHC class II serves to present peptides that can be derived from

exogenously acquired proteins or protein complexes (Figure 2). Such material can be taken up through several endocytic mechanisms, including phagocytosis, (macro)pinocytosis and receptor-mediated endocytosis. Within the endosomal pathway, the intracellular pathways of endocytosed material and newly synthesized MHC class II converge optimally in late endosomal structures [43]. In professional APCs these endosomal/lysosomal organelles are also referred to as MHC class II compartments or MIICs [34, 44, 45]. Conditions within these compartments are ideal to unfold and hydrolyze endocytosed proteins into peptides that can immediately be loaded onto MHC class II. Most MHC class II molecules are however loaded with "self" peptides, deriving from degraded self proteins that are abundantly present within the endocytic tract. In immature DCs, most of the peptide loaded MHC class II is transferred to and degraded in lysosomes relatively fast after formation. This ensures low exposure of MHC class II, loaded with self-peptides, to T cells in the absence of danger signals. Limited exposure of peptide loaded MHC class II (pMHCII) in the absence of costimulatory signals may provide cues that support tolerance. In activated DCs however, pMHCII complexes are efficiently transferred from MIICs via transport tubules and vesicles to the plasma membrane, for potential interactions with $CD4^+$ T cells.

DC – T cell interaction

Adaptive immune responses require activation of naïve T cells. T cells originate in the bone marrow but differentiate to naïve T cells in the thymus, hence their name. They can be divided into two major subsets, T cells expressing the CD8 coreceptor ($CD8^+$ T cells, also referred to as cytotoxic or killer T cells) and T cells expressing the CD4 coreceptor ($CD4^+$ T cells or T helper cells). T cells bear a receptor complex at the plasma membrane, the T cell receptor (TCR), which is unique for each naïve T cell. Each TCR is able to recognize a specific peptide-MHC (pMHC) complex on the APC surface. Only after recognition, the TCR transmits a signal that activates the naïve T cell to proliferate and differentiate into an activated T cell. Engagement of the TCR with MHC class I is restricted to $CD8^+$ T cells while interaction with MHC class II is constrained to $CD4^+$ T cells. In vivo,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α
β
peptide loaded MHC class II complex
MHCII-Ii
MHCII-CLIP
MHCII-self peptide
HLA-DM
ubiquitinated MHCII
proteases
self-protein
VPS4
ESCRT

Figure 2 (left). Model for MHC class II trafficking in immature DCs
After synthesis in the ER, MHC-Ii complexes are transferred via the Golgi and plasma membrane to the endocytic pathway with the aid of sorting signals that are contained within the cytoplasmic domain of Ii. In the endosomal system, Ii is progressively degraded by lysosomal hydrolases, eventually removing the Ii-encoded sorting signals. The last remaining fragment of the Ii, CLIP, is substituted from MHCII by other peptides that are present within the endosomal lumen, in a process that is facilitated by DM. Once Ii is removed, MHCII can also be ubiquitinated by the E3 ligase MARCH I. The ubiquitin-tag on now peptide loaded MHCII drives its sorting to ILVs, probably through interactions with ESCRT. pMHCII may also escape to the plasma membrane after which it is endocytosed and transported back to the endosome. This pathway may also be facilitated by ubiquitination of MHCII. pMHCII that is sorted to ILVs is ultimately degraded after fusion of the MVB with the lysosome. Some MHCII may be transferred from the MVB delimiting membrane to the lysosomal delimiting membrane, and from there to the plasma membrane to present lysosomally generated peptides. Boxes show a detailed cartoon of a nonameric MHCII-Ii $(\alpha\beta Ii)_3$ complex and a peptide loaded MHCII complex, as situated in a membrane. Also indicated are the $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ domains of the α and β chains of MHCII.

the architecture and local environments within the lymphoid tissues support T cells in scanning and discriminating pMHC complexes on the surface of locally present APCs [46]. The chances for TCRs to interact with DCs expressing their cognate pMHC are increased by high cell contact dynamics: T cells are capable of interacting with up to 5000 DCs in 1 hour [47, 48]. These brief contacts are also referred to as immunological kinapses. The efficiency of T cell activation is further illustrated by observations that as few as 10 specific pMHC complexes, among the high variety of pMHC expressed by a single APC, was already sufficient to initiate a T cell response [49].

Initial contact between T cells and APCs is largely driven by the interaction between intercellular adhesion molecule 1 (ICAM-1) on the APC and the integrin lymphocyte function-associated antigen 1 (LFA-1) on the T cell. Subsequent cognate interactions between TCRs and pMHC complexes induce a relatively long-lived interface, or immunological synapse (IS), between the T cell and the APC [50]. The IS adapts to a bull's eye pattern with a central part that is defined as the central supramolecular activation complex (cSMAC) that concentrates TCR-pMHC clusters. The ring-shaped area that surrounds the cSMAC is defined as the peripheral SMAC (pSMAC). The pSMAC is enriched in interacting LFA-1 and ICAM-1 complexes, provides positional stability and enhances the efficiency of antigen detection [51]. A region outside of the pSMAC, the distal SMAC

(dSMAC), displays an abundance of the tyrosine phosphatase CD45 on the side of the T cell [52]. Within seconds after cognate interaction, microclusters of TCRs form in the periphery of the IS and move to the cSMAC. Cluster formation induces Lck-mediated phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR cytoplasmic domains, thereby initiating the activation of the T cell [53]. Upon arrival at the cSMAC, signaling is terminated by dissociation of the signaling molecules followed by internalization and lysosomal degradation of TCRs [54]. TCR signaling is however sustained through continuous formation, transport and degradation of the TCR clusters during the life-span of the IS [55]. Costimulatory signaling through interaction of the costimulatory receptor CD28 on the T cells with the costimulatory ligands CD80 (B7-1) and CD86 (B7-2) on the APC is also initiated at the TCR clusters. They are transported with the clusters towards the cSMAC but separate from the TCR at its border, generating an annular cluster around the cSMAC [56]. The secluded space that is formed between a T cell and the APC by the IS, is used for the containment of locally secreted soluble molecules (e.g. cytokines, lytic enzymes) that in this way only affect interacting and not neighbouring cells. Likewise, relevant transmembrane molecules like the TCR and IFN-γ receptor but also MHC class II molecules are transported in a polarized fashion either by the secretory or endosomal recycling pathways to the IS [36, 57-59].

Cognate interaction of MHC class I or II with the T cell receptor is a crucial step for the activation of naïve T cells and the first step towards an adaptive immune response against foreign antigens. Once a T cell has received sufficient additional signals through interactions with costimulatory molecules and cytokines from the APC it will multiply itself in a clonal manner. Activated $CD8^+$ T cells acquire a cytotoxic capacity, enabling them to kill cells expressing cognate pMHC class I complexes at their cell surface. In contrast, differentiation of $CD4^+$ T cells leads to a number of effector T cells with a variety of functions. Among these are the "T helper cells" which provide stimulatory signals in the cellular immune response (Th1) by activating $CD8^+$ cytotoxic T cells (license to kill) and macrophages. Other $CD4^+$ T cells provide stimulatory signals (Th1 and Th2) for the activation and antibody class switching of B cells in the humeral response. Another subset of helper cells is formed by the Th17 cells, owing their name due to the fact that they can produce IL-17 upon activation but not IFN-γ

or IL-4. Th17 cells act early in the adaptive immune response against bacteria, probably by stimulating the neutrophil response against such invaders [60]. $CD4^+$ T cells can also become committed to a regulatory phenotype (Treg) which are immune-suppressive, providing tolerance and preventing autoimmunity [4, 61-63].

Cross-presentation

While MHC class I is predominantly loaded with peptides that are produced from cytosolic proteins, MHC class II is loaded with peptides that are generated from exogenous antigens in endosomes after endocytic uptake. In DCs, the distinction between these sources of antigen for binding to MHC molecules is however not strict. Peptides from endocytosed material can also be presented via MHC class I, a process referred to as "cross-presentation" [64-66]. Cross-presentation is primarily a feature of DCs and is essential for the initiation of MHC class I restricted immune responses towards antigens that are not expressed by DCs themselves in for example anti-tumor or anti-viral responses. In particular the $CD8^+$ DC subtype in mice [67] and the recently discovered human equivalent $CD141^+$ DCs [68-72] have been shown to execute cross-presentation. Despite substantial research on this topic, the exact mechanisms by which exogenous peptides are delivered to the MHC class I molecule remain elusive, although two distinct routes have been demonstrated. First, internalized MHC class I molecules may be loaded with peptides from exogenous material within endosomal compartments [73, 74]. This route is analogous to that of MHC class II [75] and in fact may employ a common chaperone, the invariant chain [76]. Second, endocytosed antigens can overcome the endosomal membrane barrier and access the cytosol for proteasomal processing [77-79] after which the TAP-dependent pathway for MHC class I peptide loading can be followed [80, 81]. There is also evidence, although debated [82], suggesting the formation of hybrid ER-endosomal compartments during phagocytosis, thus allowing direct access of phagosomally generated peptides to the ER lumen [83-88]. Taken together, cross-presentation by DCs is probably accomplished by several routes which may partly overlap [66], thus creating multiple ways for DCs to cross-present peptides.

MHC class II

MHC class II consists of a non-covalently associated complex of two type I transmembrane glycoproteins, designated as the α-chain and β-chain (Figure 2). Both chains are encoded within the MHC gene region located on chromosome 6 in human known as human leukocyte antigen or HLA, and on chromosome 17 in mouse termed H-2. The murine MHC class II genes were the first that were identified to control immune responses to a given antigen and therefore were also called Immune response (Ir) genes. Hence, the murine MHC class II genes are often referred to as I-A and I-E. The antigen or peptide-presenting MHC molecules, like MHC class I and II, are known as classical MHC molecules. The MHC also encodes structurally related molecules known as non-classical MHC molecules that do not directly present peptides themselves. Examples of these are HLA-DM and HLA-DO molecules (H-2M and H-2O in mice) which regulate peptide loading of the classical MHC molecules [4, 89].

Constitutive expression of MHC class II molecules is, in contrast to MHC Class I, mainly restricted to APCs. Other cell types can also be induced to express MHC class II by certain stimuli [90-94], e.g. by the cytokine IFN-γ. MHC class II expression by non-APCs in the absence of costimulatory molecules, e.g. by epithelial cells, can have an important role in maintaining peripheral tolerance [95, 96]. On the transcriptional level, MHC class II expression is controlled by a complex of DNA binding proteins, also referred to as the MHC class II enhancesosome [97, 98]. Activation of this complex requires association with the MHC class II transactivator (CIITA). Expression of CIITA is cytokine and cell type dependent and its activity is also post-translationally regulated by monoubiquitination and phosphorylation [99-102]. In immature DCs, binding of a quaternary complex of PU.1, IRF8, NF-κB and SP1 to the CIITA promoter ensures CIITA transcription and thus MHC class II expression. This system is counter-regulated in maturing DCs by a complex containing positive regulatory domain 1 (PRDM1), resulting in inhibition of CIITA transcription [103].

The α-chain of MHC class II has a molecular weight of 33-35 kDa and contains two sites for N-linked glycosylation. The β-chain has a slightly lower molecular weight of 25-30 kDa and contains one N-linked glycosylation site.

Both chains have a short C-terminal cytoplasmic and a single transmembrane domain. The extracellular parts consist of two domains, the α2 (in the α-chain) or the β2 (in the β-chain) which resemble immunoglobulin constant domains, and a N-terminal membrane distal α1 or β1 domain [4, 104, 105] (Figure 2). Biochemical studies provided direct evidence for the binding of peptides to the MHC class II complex [106, 107] while X-ray crystallography revealed the 3D structure of the complexed α1-α2 and the β1-β2 domains and that the α1 and the β1 associate together to form the peptide binding cleft [108]. The cleft is made up of eight strands of anti-parallel β-sheets forming the floor and by two anti-parallel α-helical strands which form the borders. The floor of the binding cleft contains pockets in which peptides can accommodate their amino acid side chains. These side chains function as "anchors" and are often found at fixed positions within MHC class II associated peptides. Peptide binding is secured with these "anchor residues" but also through hydrogen bonds distributed along the length of the peptide. Although the structures of MHC class I and II are comparable, the peptide binding groove of MHC class II can accommodate longer peptides (13-25 amino acids) in comparison to MHC class I (8-10 amino acids). In MHC class I, the clusters of residues at the ends of the groove that bind to the amino and the carboxy-terminus of a peptide are conserved and maintain a conformation that closes both sides of the groove. In MHC class II, these residues are not conserved and the ends of the groove display a more open conformation. The termini of associated peptides can therefore protrude from the cleft and binding of a peptide to MHC class II is therefore much more dependent on the composition of its backbone in comparison to MHC class I binding.

Since peptides require specific and correctly spaced anchor residues for stable binding to MHC molecules, the repertoire of peptides that can stably bind is rather limited. For immune evasion, pathogens would only have to evolve in such a way that their peptides would no longer bind MHC. Yet, our ability to launch adaptive immune responses against a plethora of pathogenic invaders is generally quite efficient. This can partly be explained by the fact that both MHC class I and II are polygenic and polymorphic. Polygenic means that the MHC contains several different MHC class I and II genes. Three pairs of MHC class II

genes exist in humans (HLA-DR, DP and DQ) and two in the murine system (I-A and I-E). Similarly, three genes encoding MHC class I heavy chain are inherited from each parent. Expression of MHC genes is codominant, all are expressed at the cell surface and they have different peptide binding abilities thereby increasing the peptide repertoire that is displayed. Another layer of diversity is added by the highly polymorphic nature of the MHC class I and II genes, for some of which more than 400 alleles are known. The polymorphic residues are mainly found in the peptide binding groove, illustrating the evolutionary need for maximizing the diversity in binding peptides. Exceptions are the MHC class II α-chains of HLA-DR and I-E which are not polymorphic.

Invariant chain

In the 1980s it became clear that $CD4^+$ T cell recognition in the context of MHC class II molecules specifically depended on active processing of proteins [109, 110] and the binding of the resulting peptides to MHC class II [106, 107] in the endosomal/lysosomal compartments. This work implied that although MHC class II-α and β chains already assemble and form peptide binding sites in the ER, they selectively capture and present peptides only after their arrival at the endocytic pathway. This enigma was resolved when it was shown that partial degradation by endosomal proteases [111] or removal [112, 113] of a MHC class II associated protein [114-119] was required before peptide binding and presentation could occur. This protein, called the invariant chain (Ii), covers the peptide binding site of MHC class II after assembly in the ER, therewith preventing premature peptide loading, and chaperones MHC class II to endosomes.

Ii is a type II transmembrane protein of about 31 kDa (p31) (Figure 2). In contrast to MHC class II, Ii is encoded outside the MHC gene region, but its expression is, like MHC class II, under the control of CIITA [120]. The Ii is monomorphic, hence its name, but multiple forms of Ii do exist in the cell as a result of alternative initiation sites and splicing. In humans, a 35 kDa Ii form (p35), which is expressed due to alternative translation initiation, contains an additional segment of 16 amino acids which includes an ER retention signal

[121, 122]. Another form of the Ii, expressed both in man and mice, includes an additional 64 amino acid sequence in its luminal domain due to alternative splicing. In humans this results in a 43 and 45 kDa form (p43 and p45) and in mice in a 41 kDa form (p41) of the Ii [123-125]. This sequence contains five cysteines and is homologues to a motif found in thyroglobulin that displays protease inhibitory activity [126-128]. A clear difference in MHC class II chaperoning functions of the Ii forms has not been found in vivo [129-131] but the p41 isoform has been shown to act as a chaperone for the endosomal protease cathepsin L [132] and it also has the ability to enhance antigen presentation [133]. Ii has both N-linked and O-linked glycosylation sites and can be modified by phosphorylation, which may regulate its intracellular transport [134-136].

After its synthesis in the ER, Ii forms trimeric complexes in which all forms of the Ii can be incorporated, although the p31 and p35 form are most prominently present [121, 137]. Trimerization of Ii chains is promoted by intramolecular associations of transmembrane and luminal domains [138-140]. Through a stepwise association of MHC class II-$\alpha\beta$ heterodimers with Ii trimers, a nonameric complex is formed in the ER [121] (Figure 2), in a process that is facilitated by the ER resident molecular chaperone calnexin [141-144]. Formation of the nonameric $(\alpha\beta Ii)_3$ complex facilitates its egress from the ER and transfer to the Golgi apparatus where it undergoes complex glycosylation [145-147].

Association of MHC class II with Ii stabilizes the $\alpha\beta$-dimer and absence of Ii leads to accumulation of misfolded class II molecules in the ER [148, 149]. Ii binds the MHC class II peptide binding groove with a region in its luminal domain which is called CLIP for class II-associated invariant chain peptide [150-154]. Normally, Ii is synthesized in excess over MHC class II, ensuring that all molecules can associate with Ii [122, 145, 155, 156]. In this way, the Ii possibly prevents binding of other potential substrates to MHC class II in the ER that expose the same sequences that normally bind in endosomes [113, 157]. As discussed above, the ER contains peptides that have been transferred from the cytosol into the ER by TAP for binding to MHC class I. In the absence of Ii, TAP transferred peptides could potentially bind to MHC class II. It has been shown however, that binding of such peptides is inefficient [158-162].

The endosomal system

Cells continuously internalize plasma membrane components together with extracellular material through multiple endocytic mechanisms [163, 164]. One route is provided by clathrin-mediated endocytosis, a pahway that allows selection of integral membrane receptors and associated ligands into clathrin-coated pits through recognition of their cytoplasmic domains by adaptor proteins. Clathrin-independent pinocytic pathways are less specific but may perform up to 50% of total endocytosis [165] and include the caveolar, ARF6 and the GLIC/GEEC driven endocytosis pathways [166, 167]. It is estimated that mammalian cells internalize and recycle the equivalent of 50-180% of their cell surface every hour. In case of macrophages, the amount of endocytosis can even surmount up to 30% of cell volume per hour [168]. To maintain a membrane balance there is a need to return the majority of the internalized membrane components back to the plasma membrane, a task which is coordinated by the endosomal system.

The endosomal system consists of pleiomorphic intracellular organelles that are intimately connected with the plasma membrane, trans-Golgi network (TGN) and lysosomes (Figure 3). The lysosome can be considered as an endpoint of the endocytic pathway where many endocytosed cargo proteins are degraded. Despite the high plasticity of the endosomal organelle, a rough classification can be made based on morphology, luminal pH, functional properties, the relative abundance of certain proteins or lipids and the access times for internalized markers to reach a particular endosomal station [169]. The early endosome (EE) is considered to be the primary accessed endocytic structure, is transient and first formed by fusing transport vesicles, is located in the periphery of the cell and has a vesicular-tubular morphology. EEs can be subdivided into the sorting endosome (SE) and the recycling endosome (RE) or endocytic recycling compartment (ERC). With time, SEs incorporate incoming fusing endocytic vesicles and adapt to a more vacuolar morphology [170, 171]. At the same time, SEs recycle most of their acquired membrane components to the plasma membrane either directly or via tubular REs. Due to its progression in space and time the organelle is then also termed the late endosome (LE). Subsequent fusion of the LE with the lysosome [172] results in a hybrid organelle, referred to as the endolysosome, where most of the degradation of the

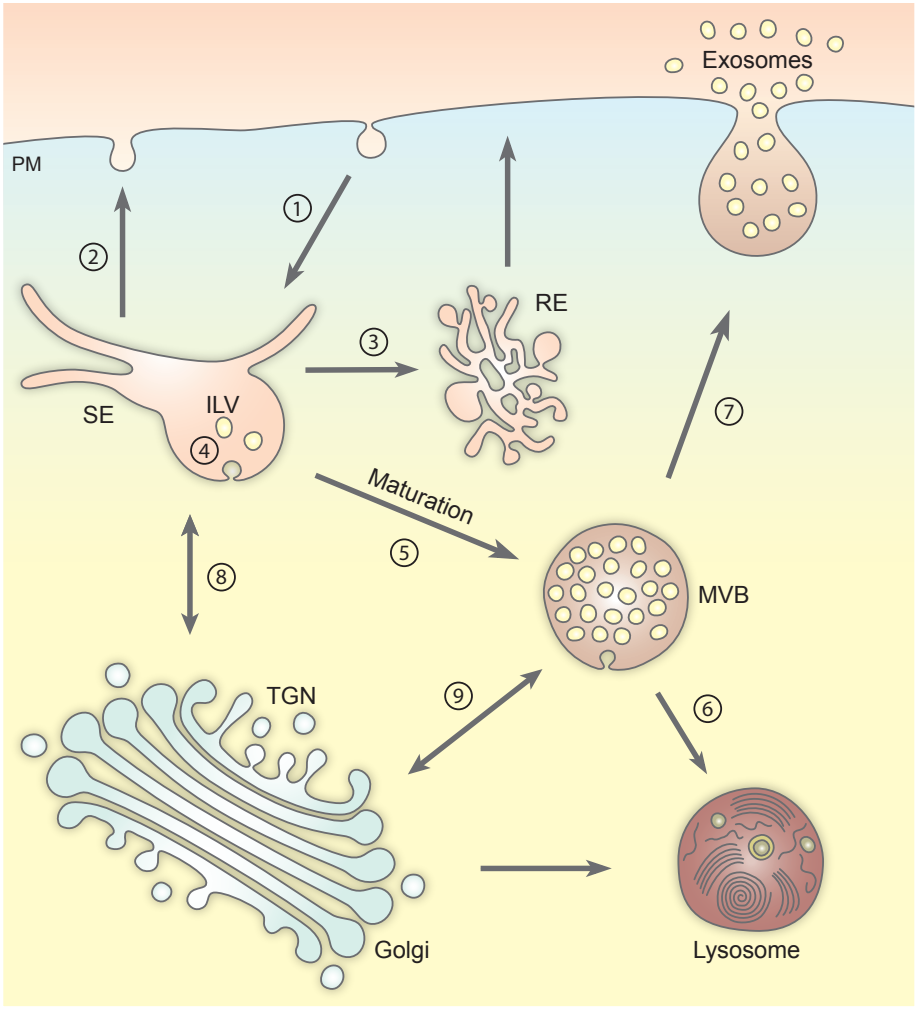

Figure 3. The endosomal system
Membrane receptors, ligands and solutes are endocytosed through a variety of mechanisms (1) and transported to sorting endosomes (SEs). Most membrane components recycle back to the plasma membrane (PM) by default, either directly from the SE (2) or via the recycling endosome (RE) (3). Solutes and dissociated ligands are largely retained in SEs. Some transmembrane proteins are selectively incorporated into vesicles that bud inward into the endosomal lumen, referred to as intralumenal vesicles (ILVs) (4). SEs mature into late endosomes while accumulating ILV (5). Endosomes that have accumulated many ILVs are also referred to as multivesiculair bodies (MVBs). Fusion of MVBs with lysosomes (6) will ultimately allow the degradation of ILV and their contents. Alternatively, some MVBs may fuse with the plasma membrane (PM) instead (7). This results in the extracellular release of their ILVs, which are now called exosomes. Endosomes continuously exchange proteins with the trans-Golgi network (TGN) (8,9). Cargo exchange between endosomal compartments, PM and TGN is mediated via transport vesicles.

endosomal contents takes place.

Newly formed SEs continue to receive endocytic vesicles for about 5-10 minutes. The lumen of SEs is mildly acidic (pH 5,9-6,5) [173, 174], driving the release of many ligands from their receptors. The bulk of membrane proteins is rapidly directed away via tubular extensions and vesicular carriers to the plasma membrane or REs. Due to the relatively small diameter of such tubules in comparison to that of incoming vesicles, relatively little of the solute is recycled, driving condensation of the non membrane associated material. The RE is a relatively long-lived tubular organelle and considered to be a distinct section of the endosomal system to which mainly membrane cargo from the SE is delivered for further transport to the plasma membrane or the TGN. Direct recycling from the SE to the cell surface is relatively fast (t½ ~2 minutes) [175-177] whereas recycling via the RE is much slower (t½ ~10-30 minutes) [178-180]. The tubules of the SE and RE contain multiple domains that are associated with endosome specific cytosolic factors, including Rab4, Rab5, Rab11, Arf1/COPI, and retromer which provide mechanisms for molecular sorting and targeting to other destinations [164, 181, 182]. During maturation, SEs gradually decrease their exchange with the plasma membrane, decrease their pH further to 5,0-6,0 [183, 184] and start migrating along microtubules towards the perinuclear area. At this stage, the endosome also acquires increasing amounts of acid hydrolases from the TGN which gain activity due to the decreasing pH in the endosome. Another established hallmark of the transition of SEs to LEs is the conversion of phosphoinositide species (PI). PIs play a main role in membrane trafficking through recruitment and activation of specific cytosolic proteins at the cytosolic leaflet of the membrane [185]. On endosomal membranes, both PtdIns(3)P and PtdIns(3,5)P_2 are present. Indicative of endosomal maturation is the conversion of PtdIns(3)P, mainly present on EEs, to PtdIns(3,5)P_2, mainly present on LEs and lysosomes. Endosome maturation is also displayed by a relatively defined switch from the EE association of the Rab5 GTPase to Rab7 binding at LEs [186, 187]. Already at the SE stage, the vacuolar sections start to generate intraluminal vesicles (ILVs) which increase in number during the transition towards the LE. During this stage in endosome maturation, membrane associated cargo destined for degradation is targeted to and concentrated on the ILVs in the endosomal lumen, giving rise to an eminent multivesicular morphology. This

type of LE is often referred to as the multivesicular body (MVB) or multivesicular endosome (MVE) [188]. Incorporation in ILVs terminates ligand induced signaling of many plasma membrane receptors, such as receptor tyrosine kinases and G protein-coupled receptors [189]. In addition, it is suggested that this process is used to deplete certain cytosolic proteins which are bound to cytoplasmic domains of membrane proteins that are targeted to ILVs [190]. The next stage in endosomal development includes progressive degradation of the MVB content, especially after fusion of the MVB with the lysosome. As an alternative destiny, MVBs can fuse with the plasma membrane thereby releasing their ILVs as exosomes in the extracellular space [191, 192].

Ubiquitin mediated sorting

Endocytosis of many plasma membrane proteins and their sorting into the MVB is regulated by ubiquitination [193, 194]. In this process, ubiquitin molecules are post-translationally and covalently attached at one (monoubiquitination) or more sites (multiubiquitination) at the cytosolic domain of a membrane protein. Linkage occurs usually, but not always, between the C-terminus of the ubiquitin molecule and specific lysine residues of the substrate protein. Ubiquitin itself can also be subject to ubiquitination. It contains seven lysine residues to which other ubiquitin moieties can be attached, creating an oligo- or polyubiquitin chain through distinct linkages. Ubiquitination is accomplished through sequential actions of an ubiquitin activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligating enzyme (E3). The E3-ligase usually determines the substrate specificity. The E3-ligases implicated in endocytic membrane trafficking are the Nedd4-family of HECT (homologous to E6 carboxy terminus) domain containing E3 ligases, the RING (really interesting new gene) domain containing E3 ligases (e.g. Cbl family) and the MARCH (membrane-associated RING-CH) family. The HECT and RING domains both constitute the binding site for the E2 enzyme. Protein ubiquitination can be counteracted by the action of a large family of deubiquitinating enzymes (DUBs) which can remove conjugated ubiquitin chains. As such, the ubiquitin tag as a sorting signal is regulated by the modular, transient and transferable features of

the system. The ubiquitin system also plays a major role in many other cellular processes, like transcriptional regulation and degradation of cytosolic proteins by the proteasome [195]. In clathrin-mediated endocytosis, two clathrin associated proteins, Eps15 and Epsin, are the best characterized ubiquitin binding adaptors. They can bind ubiquitinated cargo at the plasma membrane through their ubiquitin-interaction motifs (UIMs) and sequester the cargo at the site of clathrin coated vesicle formation [196]. Endocytosis of ubiquitinated proteins by a clathrin-independent route is also reported [197] but not firmly established.

At the endosomal system, ubiquitination driven cargo sorting and formation of ILV is facilitated by the endosomal sorting complex required for transport (ESCRT) [198-200]. The components of this system have been distilled out of the so-called "class E" vacuolar protein sorting (vps) genes that, when knocked out in yeast, displayed abnormal endosome morphology and inhibited cargo delivery to the vacuole [201]. ESCRT components are also recognized for their role in cytokinesis, viral budding [202-204] and autophagy [205]. These processes have in common that they all exploit a general functionality of the ESCRT machinery that involves membrane budding and fission away from the cytosol. The ESCRTs comprise a pathway of five distinct complexes, ESCRT-0, -I, -II, -III and Vps4 which are all well conserved from yeast to man. For simplicity, the mammalian nomenclature for the ESCRT constituents is used in the following part of this chapter.

ESCRT-0 consists of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducer adaptor molecule) subunits that form a heterodimeric complex. Hrs contains a FYVE domain, enabling the complex to bind to PtdIns(3)P, providing specific recruitment to endosomal membranes [206]. Hrs can also bind clathrin via its clathrin box motif and Hrs and STAM are both able to bind ubiquitin with their UIMs (ubiquitin interaction motifs) and VHS (Vps27/Hrs/STAM homology) domains, making ESCRT-0 an excellent receptor for ubiquitinated cargo at the endosomal surface. This initiation phase of the ESCRT pathway can be observed by electron microscopy as a characteristic flat lattice at the cytoplasmic face of the endosome [207-209]. ESCRT-I is a heteromeric complex of four subunits, Tsg101 (tumor susceptibility gene 101), Vps28, Vps37 and hMvb12. Tsg101 has an UEV (N-terminal Ubiquitin E2 Variant) domain that can bind ubiquitinated cargo and the P(S/T)XP motifs

of ESCRT-0. An additional UBD (ubiquitin binding domain) is present in hMvb12 and a basic helix in the N-terminus of Vps37 stabilizes membrane binding of ESCRT-I. In turn, ESCRT-I interacts with ESCRT-II, a heterotetramer complex consisting of EAP30 (ELL-associated protein of 30 kDa), EAP45 and two subunits of EAP20. They interact via the GLUE (GRAM like ubiquitin-binding in EAP45) domain and the C-terminus of Vps28. The GLUE domain in ESCRT-II is also responsible for its binding to ubiquitin and PtdIns(3)P, again promoting the recruitment and endosomal membrane localization of ubiquitinated cargo. ESCRT-0, -I and -II all form stable cytoplasmic complexes. In contrast, ESCRT-III is assembled on the endosomal membrane out of four core subunits denoted as CHMPs (charged multivesicular body proteins). CHMP2, 3, 4 and 6 exist in the cytoplasm as inactive monomers that lack ubiquitin binding activity but have a basic patch at their surface that binds strongly to acidic membranes. Binding of the ESCRT-II component EAP20 to CHMP6 initiates the recruitment and homo-oligomerisation of CHMP4 on the membrane. CHMP4 oligomerization is believed to be stopped by sequential binding of CHMP3 and CHMP2. ESCRT-III assembly results in a spiral structure that encircles cargo already sequestered by the upstream ESCRTs, and supports membrane budding and vesicle formation. Ubiquitin is recycled from the sorted molecules at this point by the action of AMSH (associated molecule with SH3 domain of STAM), a DUB that interacts with ESCRT-III but also with ESCRT-0. Alternatively, another ESCRT-0 interacting DUB called UBPY (ubiquitin peptidase Y) is involved in salvaging ubiquitin molecules from degradation. Information whether also other DUBs are involved or regarding substrate specificity for DUBs is not available to date. In contrast to the other ESCRTs, the exact stoichiometry of the ESCRT-III polymer remains elusive but its configuration is very stable. Its disassembly requires the ATP-hydrolysis driven action executed by a fifth ESCRT unit, the class I AAA ATPase Vps4 complex [210]. Monomeric Vps4 units assemble as a dodecamer of two hexameric rings with a central pore. Binding of an accessory molecule, LIP5 (lyst-interacting protein5), enhances Vps4 oligomerization, ATPase activity and ESCRT-III binding. Both LIP5 and Vps4 contain MIT (microtubule-interacting and trafficking) domains that bind to MIMs (MIT-interacting motifs) on the ESCRT-III units. Vps4 activity and binding to ESCRT-III are required for normal MVB formation but the mechanism by which ESCRT-III dissociation from the

membrane is regulated is still obscure. It has been postulated that disassembly is facilitated by "pulling" ESCRT components through the central pore of the Vps4 structure [211]. This would however require a subsequent refolding step for the complete recycling of ESCRTs. In summary, the ESCRT components exhibit multiple UBDs, membrane binding and deformation activities that enable the sequestration of ubiquitinated cargo into ILV.

In addition to ESCRT-dependent cargo selection and ILV formation also ESCRT-independent mechanism(s) appear to exist [212, 213]. These may require the presence of certain lipids such as lysobisphosphatidic acid (LBPA) and ceramide [214, 215]. Such data may also suggest that the ESCRTs may not directly drive vesicle formation but mainly orchestrate a proper composition and efficiency of lipid-driven membrane deformation and ILV formation. This model is supported by the observation that lipids enriched in lipid rafts are also found to be enriched in exosomes, which are believed to be the secreted form of ILVs. The membrane binding molecules anexin-1 and 2 have also been implicated to play a role in MVB biogenesis [216-218] and also tetraspanin proteins may contribute to MVB formation [219, 220]. As their name implies, tetraspanins span the membrane four times and are palmitoylated, a post-translational modification that stimulates lateral partitioning into protein/lipid domains [221, 222]. Through homotypic and heterotypic interactions they are able to organize into tetraspanin-enriched microdomains (TEMs) together with cholesterol and other membrane proteins like integrins or receptors [223, 224]. TEMs may thus facilitate the inclusion of a variety of cargo proteins into ILVs via microdomain-mediated budding [225, 226]. Together, these studies suggest that additional MVB pathway(s) may exist, which can have different functional properties, e.g. for secretion of exosomes.

Processing of Invariant chain and antigen

The endolysosomal system contains a variety of acid hydrolases, including proteases, lipases and nucleases. One major group of endolysosomal proteases are the cathepsins, consisting of at least 15 members. Most of these are ubiquitously expressed although some have more tissue or cell type specific expression profiles. Many cathepsins, but not all, are optimally active at low

pH and require processing of the precursor protein. Based on their active site, cathepsins can be subdivided into aspartic (cathepsin D, E and AEP), serine (cathepsins A and G) and the lysosomal cysteine cathepsins [227]. In APCs, cathepsins participate both in Ii degradation and antigen processing [228]. Reduction of disulphide bonds, performed by the enzyme GILT (γ-interferon induced lysosomal thio reductase), can be an additional important step in antigen processing because it leaves the antigens more susceptible for further proteolysis [229]. Protease activities, pH and reducing conditions are not uniform throughout the endosomal system, creating multiple processing environments that contribute to the generation of a plethora of antigenic peptides [230-232].

After arrival at the TGN, newly synthesized MHC class II $(\alpha\beta Ii)_3$ complexes are directed to the endocytic system by two classical di-leucine sorting motifs [233] present in the Ii cytoplasmic domain [234-237] (Figure 2). Both motifs interact with the sorting protein complex AP1, which is a trans-Golgi network adaptor, and AP2, which is a plasma membrane adaptor [236, 237]. It is well established that Ii controls the transfer of MHC class II $(\alpha\beta Ii)_3$ to endosomes, and both a direct a direct route from the TGN [238-241] and indirect transport via the plasma membrane [242-246] are apparent. Since the peptide repertoire presented by MHC class II is generated throughout the endolysosomal system it is plausible that entry of the $(\alpha\beta Ii)_3$ complex at different sites of the endocytic tract may help to broaden the repertoire of bound peptides. Within the endocytic pathway, MHC class II is particularly enriched in LE and endolysosomal compartments, the before mentioned MIICs. MIICs can have a MVB and/or multilaminar morphology [247] and are positive for the late endosomal/lysosomal marker LAMP1 (lysosome associated membrane protein 1). Based on electron microscopy studies, about 60% of total MHC class II is found at these compartments in immature DCs [33, 34].

At the endosomal system Ii is removed from MHC class II by a multitude of proteases in defined sequential cleavage steps, starting at the C-terminal luminal domain, until only the CLIP fragment is left, which occupies the peptide binding groove of MHC class II [230]. Intermediate Ii degradation products ranging from 25 kDa to 10 kDa (p25-p10) could be identified, especially when proteolytic activity of endosomes was inhibited [248-250]. Processing of Ii appears to be based on redundant protease activities [251] with the exception of a requirement for Cathepsin S [252-255], and of Cathepsin L and F [256, 257] in certain cell types, for

performing the terminal cleavage of the p10 intermediate to yield CLIP. It has been proposed that Ii degradation is regulated and increased upon activation of DCs [258] but later studies proved otherwise [31, 259-261].

Peptide loading of MHC class II

In general, peptide loading of MHC class II occurs after Ii processing into CLIP. As an exception, for some MHC class II haplotypes complete processing of Ii into CLIP is not required for peptide loading [262]. In MIICs, the MHC class II-bound CLIP fragment of Ii, can be exchanged for other peptides, which is facilitated by the non-classical MHC molecule HLA-DM (H2-M in mice) [263-267] (Figure 2). Although HLA-DM (DM) is structurally similar to MHC class II, it lacks the ability to bind peptides. Depending on the binding state between a peptide and MHC class II, DM associates with MHC class II and provokes dissociation of low affinity peptides (such as CLIP), after which another peptide can bind [268]. During this exchange of peptides, the MHC class II binding groove is temporarily empty, a state which is rather unstable and prone to aggregation [269, 270]. DM prevents this aggregation and stabilizes empty MHC class II molecules, keeping them in a peptide receptive state [271-273]. This important editing process catalyzed by DM favors the generation of high-affinity peptide MHC class II complexes for presentation to T cells. Another non-classical MHC molecule involved in the regulation of antigen presentation via MHC class II is HLA-DO (H2-O in mice) [274, 275]. HLA-DO (DO) is also structurally similar to MHCII, even more so than DM, and forms stable complexes with DM thereby inhibiting its peptide editing function [276, 277]. DM-DO complexes are formed directly after their synthesis in the ER and are transported as such to the endosomal system [278]. Inhibition of DM activity by DO is suggested to decrease at pH values lower than pH 5,5 [279-281]. DO thus inhibits DM function early in the endocytic pathway but allows peptide editing by DM in late endocytic compartments. Furthermore, expression of DO is down-regulated upon activation of B-cells and DCs [282-285]. This may enhance DM catalyzed peptide loading under inflammatory conditions, while DO could dampen presentation of self-antigen under naïve conditions [286].

Trafficking of peptide loaded MHC class II

As indicated above, exposure of the nonameric MHC class II $(\alpha\beta Ii)_3$ complex to endosomal proteases leads to the progressive degradation of the Ii (Figure 2). In addition to peptide loading, this also results in the release of MHC class II-$\alpha\beta$ dimers and removal of the Ii-encoded sorting signals from the complex. From this point on, pMHCII may either be sorted to lysosomes for degradation or be transported to the cell surface [287, 288]. In immature DCs, most of the MHC class II resides at LEs, which are not typical recycling compartments. Nevertheless, both Ii associated MHC class II and pMHCII can traffic through a recycling route with kinetics that are in agreement with SE mediated pathways [289-291]. In a recent integrated siRNA screen on MHC class II expressing melanoma cells [292], several factors affecting transfer of MHC class II to the plasma membrane have been identified. These include the GTPase ADP-ribosylation factor-like protein 14 (ARL14). This GTPase recruits the effector protein ARF7EP to the MIIC, and acts as a receptor for the actin-based motor protein myosin 1E, putatively driving transport of MHC class II from MIIC to the plasma membrane during DC maturation.

In maturing DCs, transport of MHC class II is mediated via membrane tubules radiating out from the MIIC towards the plasma membrane which also depend on an intact microtubule network [34-39]. MHC class II, DM and LAMP1 are not actively recruited into these tubules but rather enter this pathway by default [34]. This is in agreement with the finding that traffic of MHC class II to the cell surface does not rely on sorting information encoded by its cytosolic domains [288]. After arriving at the cell surface, pMHCII molecules may be endocytosed and recycle through early endocytic compartments [291, 293, 294]. Such a pathway provides an opportunity for MHC class II molecules to acquire and present antigens that differ from those found in the late endosomal compartments, thereby contributing to the diversity in antigen presentation [289, 295-297].

It is well established that newly synthesized MHC class II-Ii complexes that are deposited on the plasma membrane, are rapidly taken up by clathrin mediated endocytosis and that this process is largely driven by the two di-leucine motifs encoded in the cytoplasmic domain of the Ii [245, 246]. The mechanism for endocytosis of pMHCII is less well understood. Truncation of both

the cytosolic domains of the α- and β-chain negatively affected internalization [289]. More specifically, a conserved di-leucine motif in the cytosolic domain of the β-chain has been proposed to play a role in pMHCII endocytosis [298, 299]. Theoretically however, this motif does not meet the requirements for interaction with clathrin adaptors as it lacks essential upstream negatively charged amino acids [233]. We and others have shown that endocytosis of MHC class II is facilitated by the ubiquitination of the cytoplasmic domain of the MHC class II beta chain [300, 301] (see chapter 2 of this thesis), suggesting that a clathrin mediated pathway could be involved. Other studies however, indicated that pMHCII complexes, in contrast to MHC class II-Ii complexes, are endocytosed via a clathrin and dynamin independent pathway [302]. Yet other studies observed that MHC class II is found in lipid rafts or detergent resistant membranes (DRMs) [303-305] and that endocytosis of MHC class II was severely hampered after treatment with filipin, a cholesterol sequestering agent, pointing towards the involvement of lipid rafts [306]. Thus, it can be hypothesized that pMHCII can enter the cell via the clathrin-independent carrier (GLIC)/GPI-AP-enriched early endosomal compartment (GEEC) pathway, an endocytosis route that is also used by other lipid raft associated molecules like GPI-linked proteins and cholera toxin B [166, 167].

MHC class II has besides in lipid rafts, also been described to be incorporated into the above mentioned TEMs [307-312]. Most tetraspanins contain motifs for clathrin mediated endocytosis [313] and could potentially assist the internalization of TEM-associated molecules, such as pMHCII. It must be noted however that the assays used in most of the above mentioned studies on MHC class II endocytosis did not discriminate between Ii-associated and pMHCII and/or did not correct for recycling of endocytosed MHC class II [291, 293], troubling their interpretation. In conclusion, the dynamics and mechanisms in DCs which regulate endocytosis of pMHCII remain largely undefined.

Ubiquitination of MHC class II

Expression levels of proteins at the cell surface are determined by their synthesis, kinetics of internalization, intracellular retention, recycling and degradation. Cell surface expression of MHC class II increases during DC maturation. In

correlation, the synthesis of MHC class II increases in response to maturation signals, but only temporally and is virtually absent in fully matured DCs [20, 28-32]. Yet, fully matured DCs display a very high surface expression of MHC class II, which can be explained by changes in its endocytic trafficking. Both proteolytic processing of MHC class II associated Ii and peptide loading of MHC class II are not accelerated during DC maturation [31, 259-261]. Still, maturing DCs establish a high and stable expression of pMHCII at the cell surface upon activation. The molecular mechanism behind this emerged from the observation that a highly conserved unique lysine at position 225 in the cytoplasmic tail of the MHC class II β-chain can be ubiquitinated [300, 301, 314]. In immature DCs, approximately 60% of total cellular MHC class II is located at MVBs of which ~80% was found associated with ILVs [33, 34] (see also chapter 4 of this thesis). Sorting of MHC class II to ILVs in immature DCs was severely impaired when lysine 225 was mutated (>20 fold less efficient as compared to wild-type) [301] (see chapter 2 in this thesis). Considering the crucial role of ubiquitination for the trafficking of many membrane proteins (as discussed above), it became evident that ubiquitination of MHC class II is important for its efficient sorting into ILVs, probably through association with the ESCRT machinery. In support of this, MHC class II is indeed enriched in the flat clathrin/ESCRT-0 coated lattices at MIICs [315], and we have seen an increase of MHC class II at the plasma membrane of DCs that expressed a dominant negative mutant of VPS4 (see chapter 3 in this thesis). Besides directing intracellular sorting at endosomes, ubiquitin tags on plasma membrane proteins may also provide for internalization signals. Indeed, endocytosis was slightly impaired for the MHC class II K225A mutant [300, 301]. It must be noted though, that the relative role for ubiquitination on endocytosis of pMHCII is not clear due to experimental difficulties as discussed above, and some studies could not confirm a role for MHC class II ubiquitination in endocytosis [316, 317].

In maturing DCs, ubiquitination of MHC class II, and as a direct consequence targeting to ILVs, is ceased [300, 301, 318, 319]. Prevention of entry into the lysosomal pathway leads to rerouting of pMHCII to the cell surface, possibly by default, causing its elevated expression at the plasma membrane. pMHCII complexes that are formed from newly synthesized MHC class II during DC activation become long-lived, generating a pool of stable pMHCII complexes potentially displaying antigen from the pathogenic stimulant. pMHCII that was

formed prior to DC activation is already largely and irreversibly sorted to ILV or lysosomes and thus degraded even after DC activation. MHCII synthesis is shut down only after DCs have fully matured. As a consequence, pMHCII that is stably displayed at the plasma membrane of mature DCs have preferentially been generated directly after contact with pathogens. In summary, the ability and efficiency to stably present pathogen derived antigens at the cell surface is largely regulated through ubiquitination driven sorting of MHC class II at endosomes.

Ubiquitination of MHC class II in APCs is driven by MARCH I [317, 318], a member of the MARCH E3 ligases [320] that indeed has been found associated with MHC class II in co-immunoprecipitation studies [321, 322]. DC activation signals rapidly lead to attenuation of MHC class II ubiquitination [301]. The molecular mechanism for attenuation is unclear but may either occur through down-regulation of MARCH I activity, or through an increase in MHC class II deubiquitination. DUB enzyme(s) for MHC class II have yet to be identified. Down-regulation of MARCH I transcription was demonstrated in maturing DCs [318, 323] and the half life of MARCH I is claimed to be shorter than 30 minutes [324]. Although these observations could explain in part the loss of MHC class II ubiquitination in maturing DCs, the relatively slow rate by which MARCH I transcription is down-regulated (>8 hrs after activation) suggests that additional mechanisms are responsible for the rapid interference (<1 hr) with MHC class II ubiquitination in response to maturation stimuli. Such regulation may be performed by the transmembrane molecule CD83, which is highly expressed on mature DCs. CD83 was shown to bind MARCH I via its transmembrane domain providing a mechanism to sequester MARCH I away from MHC class II [322]. Strikingly, the HSV-1 immediate early gene ICP0 which contains RING E3 Ubiquitin ligase activity was shown to down-modulate cellular CD83 in DCs as an immune evasion strategy [325]. Yet undiscovered regulatory mechanisms may additionally regulate MHC II ubiquitination. The pivotal role of MARCH I in regulating MHC class II mediated antigen presentation is further illustrated by observations that the anti-inflammatory cytokine IL-10 induces MARCH I expression [321, 322], thereby promoting MHC class II degradation at immune suppressive conditions. The central role of MHC ubiquitination in antigen presentation is also illustrated by reports on modulation by viruses [326] and Salmonella typhimurium [327]. In fact,

the discovery of viral E3-ligases that down-modulate surface expression of MHC class I, also led to the identification of MARCH ligases as regulators of immune processes, including those that determine MHC class II surface expression [314, 328].

Ubiquitination of MHC class II might involve, in addition to MARCH I, also other MARCH ligases. In MARCH I knockout mice, ubiquitination of MHC class II was absent in B cells but not completely ablated in DCs [317]. MARCH VIII, also called c-MIR, was also able to reduce MHC class II surface expression and accelerate MHC class II endocytosis when over-expressed [314], indicating overlapping specificities of these E3-ligases for MHC class II ubiquitination. MARCH I and MARCH VIII are both integral membrane proteins and localized at endosomes and the plasma membrane [320], providing the possibility that ubiquitination of MHC class II is perhaps performed by both MARCH I and MARCH VIII at physiological conditions. Also it can not be excluded that they act at distinct sites, e.g. at the plasma membrane to enhance endocytosis and at the endosomal membrane to drive endosomal sorting for degradation. Thus far though, MARCH I seems to play a dominant role in controlling pMHCII traffic in DCs [318].

As already discussed above, removal of the Ii in the endocytic system liberates the $\alpha\beta$ dimers from the $(\alpha\beta Ii)_3$ nonameric complex and allows MHC class II to be loaded with peptide. Whether peptide loading takes place at the delimiting membrane, at ILVs or perhaps at both locations, remained a difficult but intriguing question to answer. DM is an important facilitator for the peptide loading and editing process on MHC class II at LEs and is found both on the limiting membrane as well as on the ILVs of the MVB [34]. Both sites also provide access to the peptide pool generated in the MVB lumen. Peptide loading could thus occur at these two locations within MVBs. A study using a FRET assay in transfected HEK293 cells showed that in experimentally swollen MVBs, MHC class II and DM primarily interacted at ILVs [329]. These data suggested that peptide loading of MHC class II, by preference, may happen at this location. When peptide loading would occur at ILVs, a mechanism should exist for pMHCII to travel back from ILVs to the endosomal delimiting membrane from where it can reach the plasma membrane. Such a pathway, also referred to as the back or retro-fusion of ILVs, has indeed been proposed [34] to provide the membrane material necessary for the formation of the MIIC-associated tubules that are

induced in maturing DCs [35-38], and to explain the disappearance of the MVB during DC maturation. These MHC class II positive tubules in maturing DCs can however also emerge as a combinational consequence of an increase in (macro)pinocytosis and cargo synthesis (e.g. MHC class II), and interference with sorting to ILV. Furthermore, ILV back-fusion remains enigmatic and has not been recorded in live cells, although functional roles for lysobisphosphatidic acid, Tsg101 and Alix have been proposed [330]. Alternative to peptide loading at ILVs is a model in which the MHC class II peptide loading occurs at the delimiting membrane of the MVB. This model is strongly supported by several arguments. Firstly, it is reported that MHC class II can only be ubiquitinated after the associated Ii is processed down to the CLIP fragment and that ubiquitination drives its efficient sorting to ILVs [33, 301] (see chapter 2 and 4 of this thesis). This is in agreement with the observation that MHC class II failed to sort properly to ILVs in cathepsin S deficient intestinal epithelial cells [331] or antigen presenting cells [332]. Secondly, when the target lysine for ubiquitination was mutated, peptide loading did not differ from wild type MHC class II [300, 301]. Knock-in mice that express ubiquitination-deficient MHC class II also generated normal $CD4^+$ T cell and B cell populations and antibody titers in response to vaccination [333]. Thirdly, by far the majority of ubiquitinated MHC class II was found to be peptide loaded [301]. Fourthly, prevention of ILV formation by interfering with VPS4 function did not interfere with peptide loading of MHC class II (chapter 3 of this thesis). Fifthly, it was determined by quantitative immunoelectron microscopy that within MVBs, DM is rather enriched (70%) at the delimiting membrane [34]. In addition, DO was proposed to skew the localization of both DM and MHC class II within MVBs towards the delimiting membrane. The biophysical properties of the lateral interactions between DM, DO and MHC class II at the delimiting membrane would be more beneficial for peptide loading [334]. Collectively, these data imply that, also due to the high rate of DM-driven CLIP-peptide exchange, peptide loading happens already before ubiquitin is ligated as the sorting signal for ILVs. Finally, in a recent study we showed directly that MHC class II "stored" at the ILVs is not recruited to the plasma membrane in activated DCs [33]. In contrast, maturing DCs predominantly used newly synthesized MHC class II for antigen presentation. In DCs, the necessity for ILV back-fusion mechanism seems trivial and peptide loading at the endosomal delimiting membrane

would provide sufficient means for efficient presentation of antigens by mature DCs. In immature DCs, MHC class II is loaded with self-peptides and about 50% of total MHC class II is located at the ILVs. Recruitment by maturing DCs of this pMHCII-pool, which is generated before pathogen contact, seems not desirable since this would result in presentation of self-peptides at inflammatory conditions, possibly evoking autoimmunity. In this line of thought, it would rather be favorable when all pMHCII at ILVs would be destined for degradation in the lysosome.

Scope of this thesis

The important role of the MHC gene products for the generation of specific immune responses became evident in the beginning of the 1970s [335, 336] and since then, many facets of MHC class II have been characterized. In the past years we performed studies to gain more insight into MHC class II processing in dendritic cells. We focused on the sorting mechanisms of MHC class II, the degradation of its associated Ii and peptide loading at the endosomal system. In addition, we investigated the regulatory function of these intracellular processes on MHC class II surface expression and antigen presentation.

In chapter 2 of this thesis we report that MHC class II can be post-translationally modified by the addition of ubiquitin moieties at a unique lysine residue in the cytosolic domain of the β-chain. Furthermore, it is demonstrated that ubiquitination of MHC class II regulates its cell surface expression by providing a signal for efficient sorting towards degradation in the lysosome. The third chapter describes the effect on MHC class II homeostasis when the formation of ILV is hampered by over-expressing a dominant negative mutant of VPS4. We observed that interference with ILV formation did not affect MHC class II peptide loading but increased MHC class II expression by immature DCs, underlining the role of MVB sorting in MHC class II degradation. The long standing issue on whether the large amount of ILV-localized MHC class II present in immature DCs can be recruited by maturing DCs for antigen presentation is covered in chapter 4. Here we show that upon DC activation, antigens are presented mainly by newly synthesized MHC class II. In chapter 5 we report

that, in contrast to claims in existing literature, the degradation of the MHC class II associated invariant chain is a constitutive process and not up-regulated during DC maturation. We also pinpointed a possible artifact that can explain these discrepancies in the literature. In chapter 6 an experimental procedure is presented that improves antigen presentation by immature dendritic cells. We were able to show that an artificially intracellularly accumulated cohort of MHC class II molecules could be synchronously released to enhance cell surface expression of MHC class II and antigen presentation towards T cells. The strategy described in this chapter might be helpful to design immuno-tolerizing vaccines.

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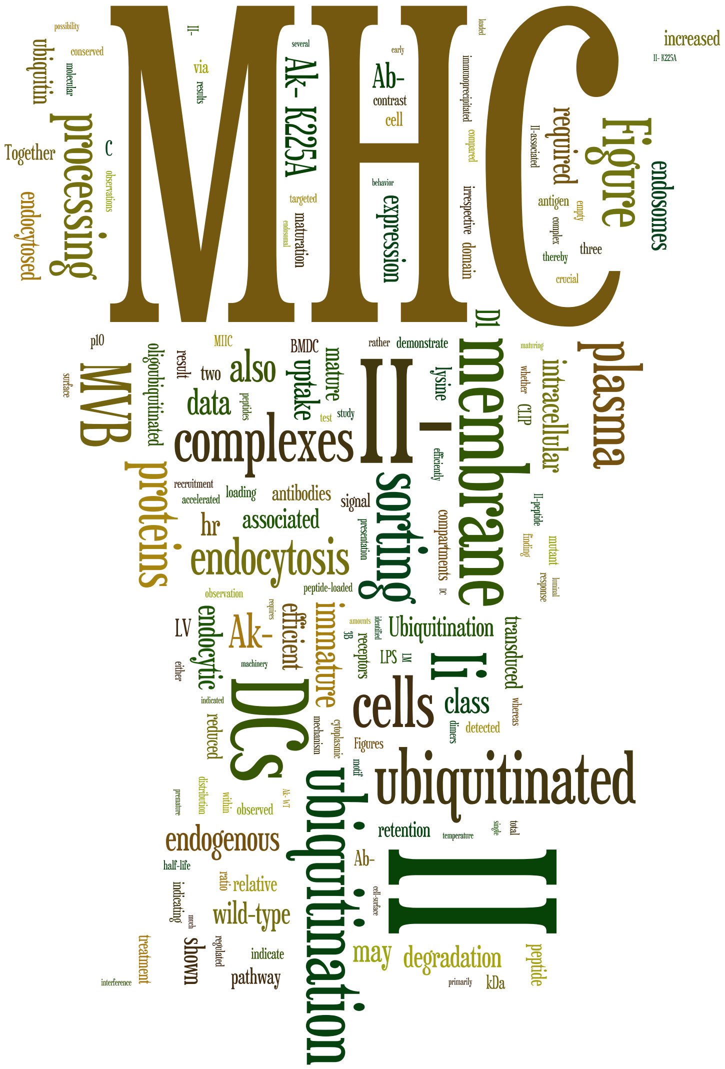
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Dendritic cells regulate exposure of MHC Class II at their plasma membrane by oligoubiquitination

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Abstract

Dendritic cells (DCs) initiate adaptive immune responses by activating T cells via cognate interactions between MHC-peptide complexes and T cell receptors. In immature DCs, MHC class II is predominantly stored in late endocytic compartments, where it has a short half-life because of degradation. In contrast, mature DCs recruit MHC class II to the plasma membrane. We here demonstrate that in immature DCs, the β-chain of MHC class II was oligoubiquitinated after proteolytic processing of the associated invariant chain in endosomes and that this modification was required for efficient endocytosis and sorting into luminal vesicles of multivesicular bodies. Ubiquitination of MHC class II was suppressed in lipopolysaccharide-activated DCs. Mutated MHC class II lacking its ubiquitination site was expressed at the plasma membrane, irrespective of DC maturation. Together, these data provide a molecular basis for the regulation of MHC class II-mediated antigen presentation by DCs.

Introduction

Dendritic cells (DCs) are crucial initiators of adaptive immune responses [1]. Toward this task, they take up pathogens from peripheral tissues by endocytic mechanisms, and internalized proteins are proteolytically processed into peptides that can be loaded onto major histocompatibility complex (MHC) molecules. Pathogen products and other danger signals induce a DC maturation program that includes effective loading of MHC class II (MHC II) with pathogen-derived peptides in endosomes and transport of the MHC II-peptide complexes to the plasma membrane. At the same time, maturing DCs migrate from peripheral tissues to secondary lymphoid organs where they can present surface-exposed MHC II-peptide complexes to $CD4^+$ T cells.

Multivesicular bodies (MVB) are specialized endosomal compartments that are composed of a single limiting membrane (LM) surrounding multiple luminal vesicles. The luminal vesicles (LV) are formed from the LM by budding

inwards, away from the cytosol, and accumulate MHC II in immature DCs [2].

MVB fuse with lysosomes, and this pathway is crucial for the downregulation and degradation of many membrane proteins and thus may also explain the abundant presence of MHC II in lysosomes [3] and its relative short half-life in immature DCs [4]. Alternatively, the MVB may fuse with the plasma membrane, releasing their LV, now termed exosomes [5]. DC-derived exosomes have been shown to have immuno-modulatory characteristics, both in vitro and vivo [5].

In mature DCs, downregulation of newly synthesized MHC II via the MVB-lysosomal degradation pathway is likely to be inhibited, thereby increasing expression of MHC II at the cell surface. Although crucial, the molecular mechanism(s) of regulation for MHC II traffic within the endocytic tract of DCs has not been resolved [6].

Both endocytosis and sorting of endocytosed proteins at MVB is often triggered by ubiquitination of their cytoplasmic domain [7]. It has recently been proposed that oligoubiquitination allows high-avidity interactions with endocytic adaptors [8, 9] that facilitate clathrin-mediated endocytosis. After uptake, ubiquitinated cargo is sorted at the MVB into LV. This depends on the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs/Vps27 and three protein complexes termed ESCRT-I, II, and III (endosomal sorting complex required for transport) that act sequentially in the sorting of ubiquitinated proteins at MVB [10].

We here report that in immature DCs, MHC II-β is oligoubiquitinated after the degradation of associated invariant chain (Ii) in endosomes. Ubiquitination of MHC II-β is inhibited in activated DCs, resulting in increased cell-surface expression. These observations indicate that antigen presentation by DCs is efficiently regulated through ubiquitination of MHC II-β.

Results

MHC II-β Is ubiquitinated in the endocytic pathway

To study whether MHC II or MHC II-associated proteins are ubiquitinated, we used both primary bone marrow-derived DCs (BMDC) and D1 cells. The latter are long-term cultured myeloid mouse DCs that have been demonstrated in many aspects to have indistinguishable behavior from freshly isolated DCs [11] and have the advantage that they can be cultured in large quantities without contaminating cell types. MHC II-αβ complexes were immunoprecipitated from cell lysates with different antibodies. From the eluate, ubiquitinated proteins were selectively reprecipitated with an antibody recognizing both mono- and oligoubiquitinated proteins, P4D1 [12]. Subsequent immunoblotting for MHC II-β or ubiquitin (Figure 1A) revealed a characteristic ladder of ubiquitinated protein bands, spaced at 8 kDa between 50 and 70 kDa. The two major bands at 50 kDa and 58 kDa were also observed in parallel silver-stained gels (Figure 1B) and analyzed by liquid chromotography/mass spectrometry/mass spectrometry (LC-MS/MS) (data not shown). Both ubiquitin and A^b-β were detected with a sequence coverage of >40%, whereas MHC II-α was not detected in these samples. The positive identification of MHC II-β and ubiquitin at the same position by immunoblotting (Figure 1A) and mass spectrometry indicate ubiquitinated MHC II-β. Immunoblot analyses for MHC II-β of total immunoprecipitated MHC II (Figure 1C) did not reveal a clear signal at 50-70 kDa, indicating that only a minor fraction of total MHC II-β is ubiquitinated. Ubiquitinated MHC II-β was detected both in D1 cells and in BMDC but not in BMDC from MHC II-β KO mice (Figure 1C). Together, these results show that MHC II-β is oligoubiquitinated in mouse DCs.

MHC II-β Is ubiquitinated after Ii processing in the endocytic pathway

Degradation of the two full-length Ii splice variants, p31 and p41, is initiated in endosomes at their exoplasmic domain, resulting in the subsequent formation of intermediate degradation products, p18 and p10. Next, processing of p10 results in the formation of the contiguous internal segment of Ii (CLIP). CLIP remains associated to the peptide binding groove of MHC II, thereby preventing binding of other peptides. Replacement of CLIP on MHC II by antigen-derived

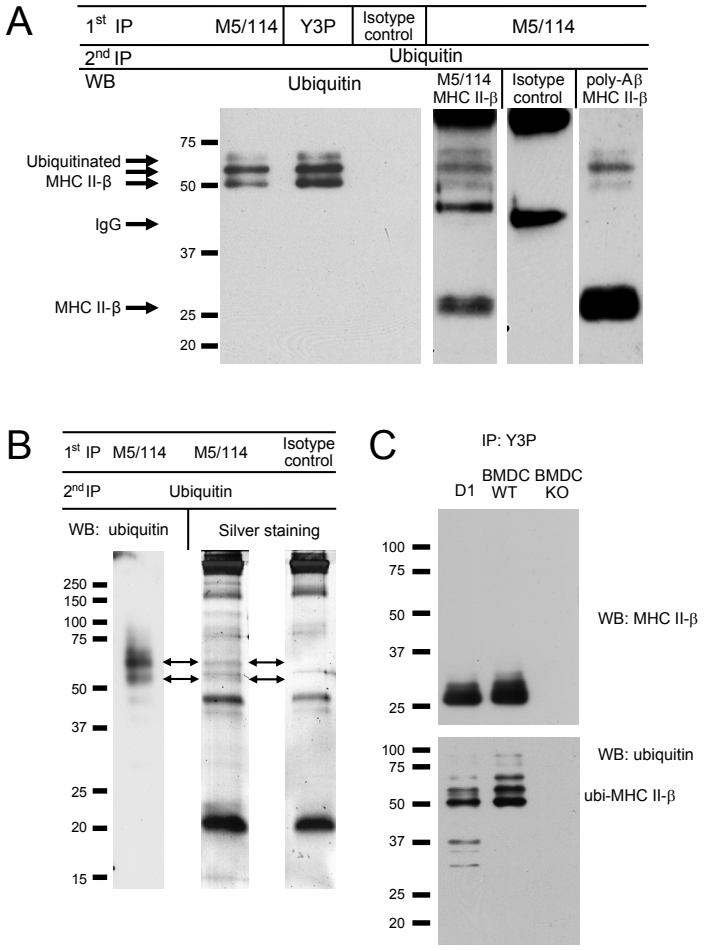

Figure 1. MHC II-β is oligoubiquitinated
(A) MHC II was immunoprecipitated from 10^6 D1 cells with M5/114, Y3P, or an isotype control antibody for M5/114 (1st IP) as indicated. Immunoprecipitates were eluted from the beads and reprecipitated with ubiquitin antibodies (2nd IP). Those precipitates were analyzed by immunoblotting (WB) for ubiquitin or MHC II-β, with either monoclonal M5/114, an isotype control monoclonal antibody, or a polyclonal antibody. The positions of ubiquitinated MHC II-β, MHC-β, and background IgG from the first IP as indicated. The figure is representative for three independent experiments. **(B)** Ubiquitinated MHC II was immunoprecipitated from 10^9 D1 cells as described above. A small aliquot (0.1%) was analyzed by immunoblotting for ubiquitin as reference (WB), and the remainder was separated by SDS-PAGE and silver stained. The bands indicated in the middle lane were identified by mass spectrometry (data not shown) and contain both ubiquitin and MHC II-β. The figure is representative for two independent experiments. **(C)** D1 cells or BMDC from WT or Ab-β KO mice were lysed and MHC II was immunoprecipitated (IP). Immunoprecipitates were eluted at 100 °C and immunoblotted (WB) for MHC II-β or ubiquitin as indicated. The figure is representative for three independent experiments.

peptides is aided by an accessory molecule, DM, and peptide loading of MHC II occurs primarily within the endocytic tract. To narrow down the intracellular location of MHC II-β ubiquitination in relation to processing of associated Ii, MHC II was immunoprecipitated with either one of three antibodies with distinct specificities: M5/114 recognizes all MHC II, irrespective of associated Ii, Y3P recognizes MHC II complexes only after p31 or p41 processing, and 15G4 recognizes the CLIP domain of Ii [13]. We further exploited the characteristic stability of MHC II complexes in SDS [14]. In the presence of SDS, MHC II-αβ dimers dissociated within 30 min at room temperature when associated with p31, p41, or CLIP, whereas prolonged incubations at 20 °C and heating to 100 °C were required to dissociate p10- and peptide-associated MHC II, respectively (Figures 2A and 2B). Collectively, these data suggested that MHC II-β is ubiquitinated only when associated to peptide-loaded MHC II (Figure 2B). This was confirmed by 2D gel analysis (Figure 2C). In the first dimension, ubiquitinated MHC II-β, which was immunoprecipitated from D1 and eluted at 20 °C, migrated as a 75-90 kDa complex. Ubiquitinated MHC II-β dissociated from these complexes at 100 °C as monitored in the second dimension. The fact that MHC II was ubiquitinated only after processing of Ii by lysosomal proteases excludes the possibility that MHC II-β was ubiquitinated in response to misfolding at the endoplasmic reticulum, for premature retrieval and proteasomal degradation. It also excludes the idea that ubiquitination of MHC II may be related to sorting at the trans-Golgi network. Given that ubiquitinated MHC II has been loaded with peptide, we anticipated that Ii processing may be required for ubiquitination of MHC II-β. To test this directly, we used the cysteine and serine protease inhibitors leupeptin and E64d to interfere with the processing of MHC II-associated Ii in endosomes and lysosomes [15, 16]. In addition, we tested the effect of MG132, a proteasome inhibitor that is known to block ubiquitin-dependent sorting of membrane proteins at MVB [17], but also inhibits lysosomal proteases [18]. All three reagents interfered with Ii processing, as indicated by the accumulation of the MHC II-associated Ii degradation intermediates, p18 and p10, and reduced MHC II peptide loading (Figure 3A). All three reagents also reduced the amount of ubiquitinated MHC II-β. Ubiquitination amounts correlated with the relative efficiencies of interference with Ii processing, indicating that Ii processing in the endocytic pathway is required for subsequent ubiquitination of MHC II-β.

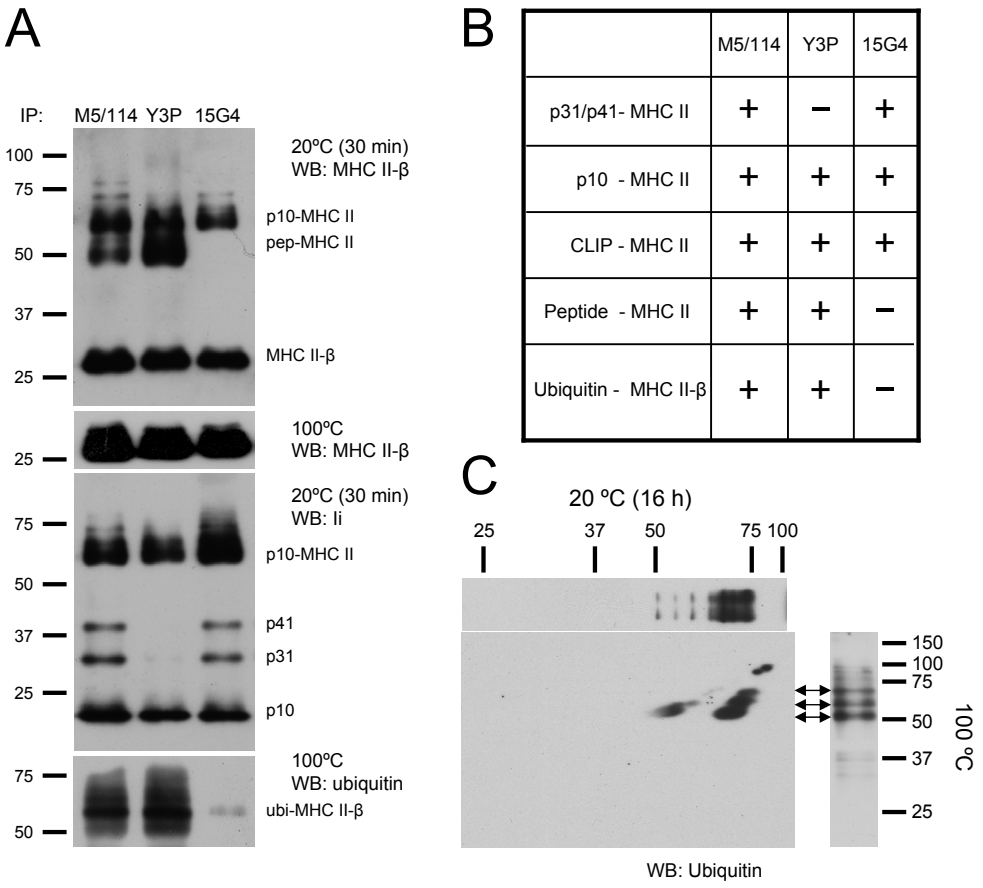

Figure 2. MHC II-β is ubiquitinated after processing of associated Ii
(A) MHC II was immunoprecipitated (IP) from D1 cell lysates as indicated by either M5/114, Y3P, or 15G4, eluted from the beads in SB at 20 ºC or 100 ºC, and immunoblotted (WB) for MHC II-β, Ii, or ubiquitin. The figure is representative for four independent experiments. **(B)** Summary of the results in (A). M5/114 precipitated all MHC II complexes, irrespective of Ii processing. Y3P precipitated all MHC II complexes except those containing full-length Ii (two splice variants, p41 and p31). 15G4 recognizes the CLIP domain of Ii and therefore precipitated all MHC II complexes except mature MHC II-peptide and ubiquitinated MHC II. **(C)** MHC II was immunoprecipitated with Y3P, eluted in SB at 20 ºC, and separated by SDS-PAGE in a first dimension. The lane was excised, incubated at 100 ºC in SB, separated in a second dimension by SDS-PAGE, and immunoblotted for ubiquitin. Appropriate immunoblots of in parallel prepared 1D lanes are projected above and to the right of the 2D blot. Ubiquitinated MHC II-β dissociated only after incubation at 100 ºC. The figure is representative for two independent experiments.

Ubiquitination of MHC II-β diminishes during DC maturation

To test whether maturing DCs ubiquitinate MHC II-β, D1 were treated with lipopolysaccharide (LPS). Maturation of DCs was indicated by increased cell-surface expression of MHC II and costimulatory molecules (data not shown) and relative increase of SDS-resistant peptide-MHC II complexes (Figure 3B). According to these criteria, maturation was not yet observed after 2 hr of LPS treatment but was clearly evident after 24 hr. In contrast, ubiquitination of MHC II-β was diminished already after 2 hr and partially reappeared from 4 hr onward, whereas the total of MHC II slightly increased during 24 hr (Figure 3B).

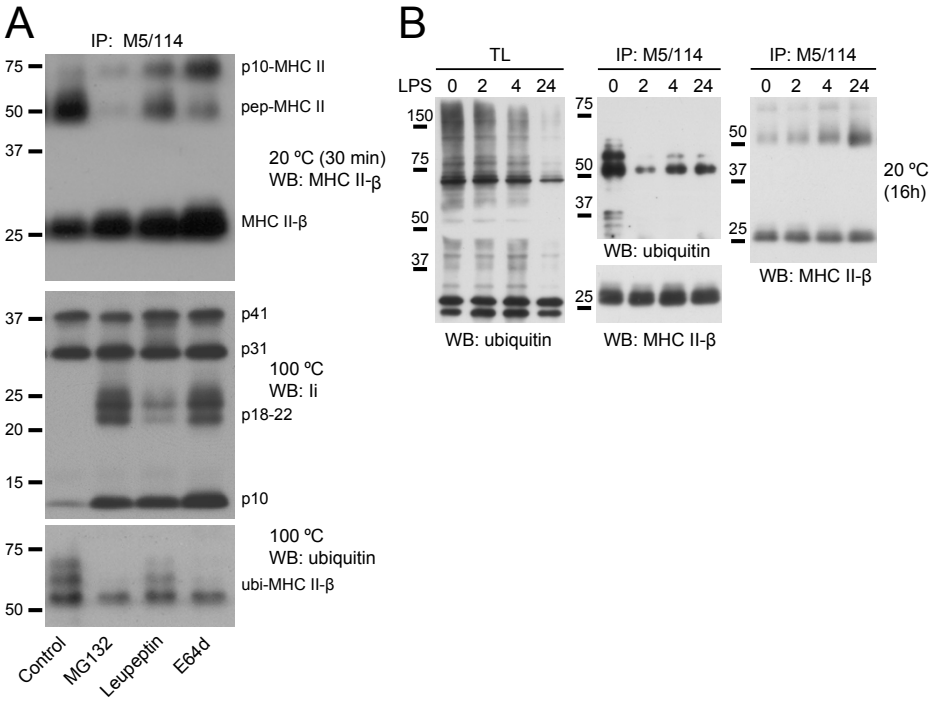

Figure 3. MHC II-β ubiquitination requires Ii processing and diminishes during DC maturation

(A) D1 cells were incubated for 3 hr in the absence or presence of MG132, leupeptin, or E64d before lysis. MHC II was immunoprecipitated (IP) with M5/114, eluted at 20 ºC in SB, and analyzed by immunoblotting for MHC II-β, Ii, or ubiquitin as indicated. **(B)** D1 cells were incubated for 0, 2, 4, or 24 hr in the presence of LPS before lysis. Samples of total cell lysates (TL) were immunoblotted for ubiquitin (left) or MHC II-β (middle bottom). MHC II was immunoprecipitated (IP) with M5/114, eluted at 20 ºC or 100 ºC in SB, and analyzed by immunoblotting for MHC II-β (right) and ubiquitin (middle top), respectively. The figures are representative for three independent experiments.

The amount of total ubiquitinated proteins in cell lysates remained constant up to 4 hr but was reduced at 24 hr (Figure 3B, left). Together, these observations indicate that ubiquitination of MHC II is selectively regulated and demonstrate a correlation between ubiquitination and intracellular retention of MHC II in immature DCs.

Intracellular retention of MHC II requires ubiquitination of MHC II-β on lysine 225

MHC II-β contains a single lysine residue at position 225, which is highly conserved among MHC II-β haplotypes and mammalian species. To test whether this residue is targeted by the ubiquitination machinery, we expressed wild-type and mutant A^k-β, in which lysine 225 was replaced by an alanine in D1 cells. A^k-β efficiently forms haplotype-mismatched heterodimeric complexes with A^b-α, and surface expression of A^k-β A^b-α dimers is as efficient as that of endogenous A^b-β A^b-α dimers [19]. This was confirmed by the formation of SDS-stable A^b-α A^k-β-peptide complexes (data not shown) and a normal subcellular distribution of these complexes in transduced D1 cells (see below). Furthermore, the distribution of A^b-α in A^b-β-deficient BMDC expressing A^k-β was indistinguishable from A^b-α in wild-type BMDC (Figure 4A), indicating efficient rescue of the distribution of MHC class II in these transduced cells. Expressing A^k-β in D1 cells allowed us to simultaneously detect two distinct MHC II complexes in the same cells via A^k-β and A^b-β-specific antibodies.

Both transduced wild-type A^k-β and mutant A^k-β^{K225A} were detected by immunoblotting immunoisolated A^b-α A^k-β complexes (Figure 4B). The signal for A^k-β^{K225A} was much higher compared to that of wild-type A^k-β, probably as a result of a prolonged half-life of A^k-β^{K225A} (see below). Transduced wild-type A^k-β but not mutant A^k-β^{K225A} was ubiquitinated, demonstrating linkage of oligoubiquitin to lysine 225. Transduced wild-type A^k-β localized together with endogenous A^b-β to intracellular DM-containing compartments (Figure 4C), again indicating that the exogenous mixed haplotype MHC II were properly assembled and targeted from the ER to endocytic compartments.

In contrast to endogenous A^b-β and wild-type A^k-β, which in immature D1 cells colocalized to the same endocytic compartments, ubiquitination-deficient A^k-β^{K225A} was primarily found at the plasma membrane (Figure 4C). These

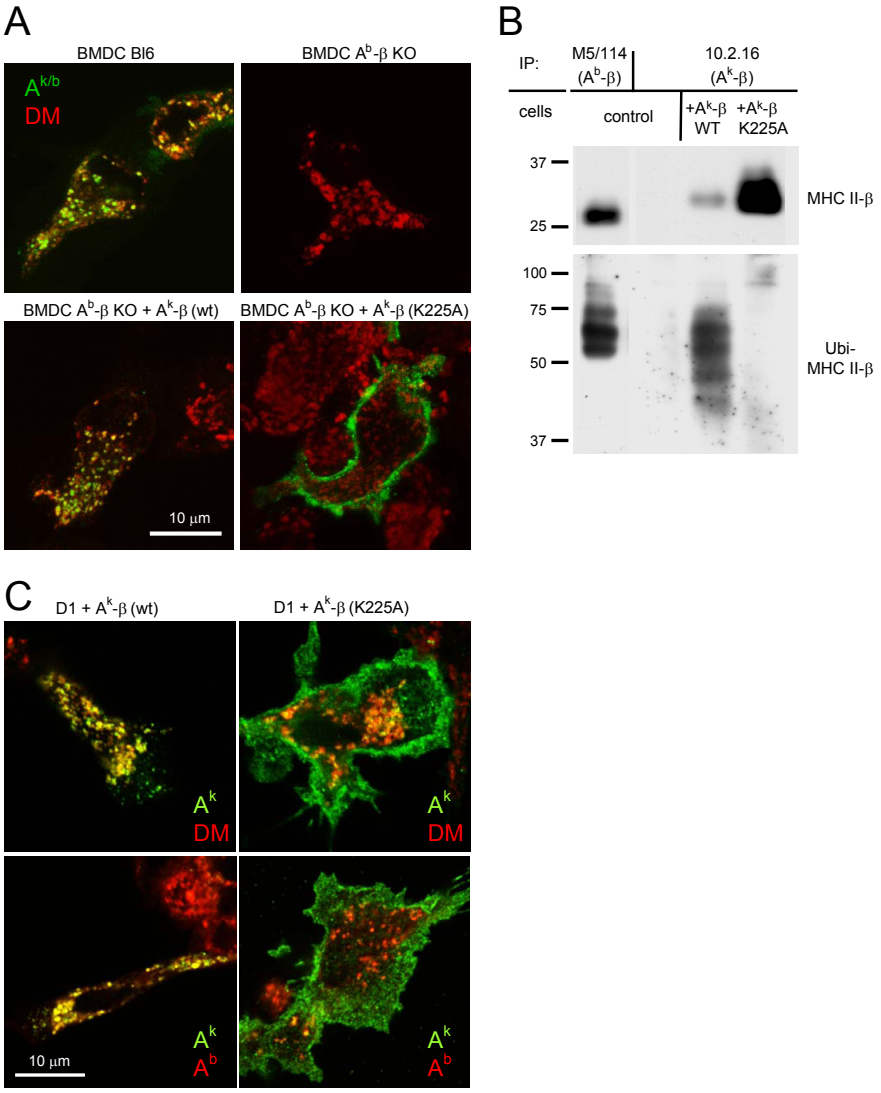

Figure 4. Ak-β^{K225A} is not ubiquitinated and targeted to the plasma membrane
(A) BMDC from either WT or A^b-β-deficient mice were transduced for either Ak-β^{WT} or A^k-β^{K225A} as indicated. Fixed cells were immunodouble labeled for DM (red) and A^b or A^k (green) as indicated and analyzed by CSLM. The figures are representative for three independent experiments. **(B)** Nontransduced D1 cells (control) or D1 transduced for either A^k-β^{WT} or A^k-β^{K225A} were lysed. Endogenous MHC II was immunoprecipitated (IP) with M5/114 and A^b-α A^k-β complexes with 10.2.16. Precipitates were eluted as indicated at 100 ºC and immunoblotted [50] for MHC II-β or ubiquitin. The figure is representative for two independent experiments. **(C)** D1 cells were transduced for either A^k-β^{WT} (left) or A^k-β^{K225A} (right) and after fixation, immunodouble labeled for A^k (green) and DM (red) (top) or for A^k (green) and endogenous A^b (red) (bottom). The figures are representative for four independent experiments.

experiments also showed that the cells did not mature as a consequence of the transduction procedure, which was confirmed by the lack of the activation marker CD86 (data not shown). After LPS treatment, A^b-β, A^k-$β^{WT}$, and A^k-$β^{K225A}$ were primarily present at the plasma membrane along with CD86 (data not shown), indicating that all constructs could be recruited from intracellular MHC II-containing compartments (MIIC) to the plasma membrane during maturation. Premature recruitment of A^k-$β^{K225A}$ to the plasma membrane was also observed in isolated BMDC from A^b-β KO mice (Figure 4A). Together, these data indicate that ubiquitination of MHC II-β occurs at lysine 225 and is required for intracellular retention of MHC II in immature DCs.

Ubiquitination of MHC II-β stimulates targeting to LV

We anticipated that the lack of intracellular retention of A^k-$β^{K225A}$ could be due to a sorting deficiency at MVB. To test this directly, we performed immunoelectron microscopy on ultrathin cryosections of immature (transduced) D1 (Figure 5). Consistent with earlier observations [2], in nontransduced D1 most of the endogenous MHC II in MVB was associated with LV (74%) rather than with the LM (Figures 5Aa and 5B). In cells transduced for A^k-$β^{WT}$, similar amounts of A^k-$β^{WT}$ (82%) and endogenous MHC II were associated with LV (Figures 5Ab and 5B). In contrast, in cells transduced with A^k-$β^{K225A}$, only little (16%) was found in association with LV, whereas the distribution of endogenous A^b-β was only slightly affected (Figures 5Ac and 5B). Together, these data indicate that ubiquitination of MHC II-β is required for sorting at MVB. Failure of ubiquitination would result in transfer by default to the plasma membrane.

Ubiquitination of MHC II-β Is required for efficient endocytosis of MHC II

In immature but not in mature DCs, MHC II is efficiently taken up from the plasma membrane by endocytosis and targeted to MIIC [20, 21]. Lack of intracellular retention of A^k-$β^{K225A}$ could thus also result from inefficient endocytosis. To study endocytosis, we took advantage of the possibility to compare simultaneous uptake of endogenous A^b and transduced mutant or wild-type A^k-β in single cells with fluorescence microscopy. Transduced D1 cells were allowed to internalize labeled monoclonal antibodies that specifically recognize A^k-β or A^b-β for 1 hr at 37 °C. As expected, in wild-type MHC II-expressing cells, endocytosed

antibodies were targeted via early endosomes that labeled for early endosome antigen 1 (EEA1) to DM-positive MIIC. Mutant A^k-β^{K225A} seemed to be delayed in the transfer from EEA1-positive to DM-positive structures (Figure 6A). To quantify relative uptake of the two haplotypes, the amounts of fluorescence in selected areas representing either plasma membrane or intracellular structures were determined in confocal sections (Figure 6B). The relative efficiencies of uptake were then calculated as a ratio of the relative fluorescence intensities at

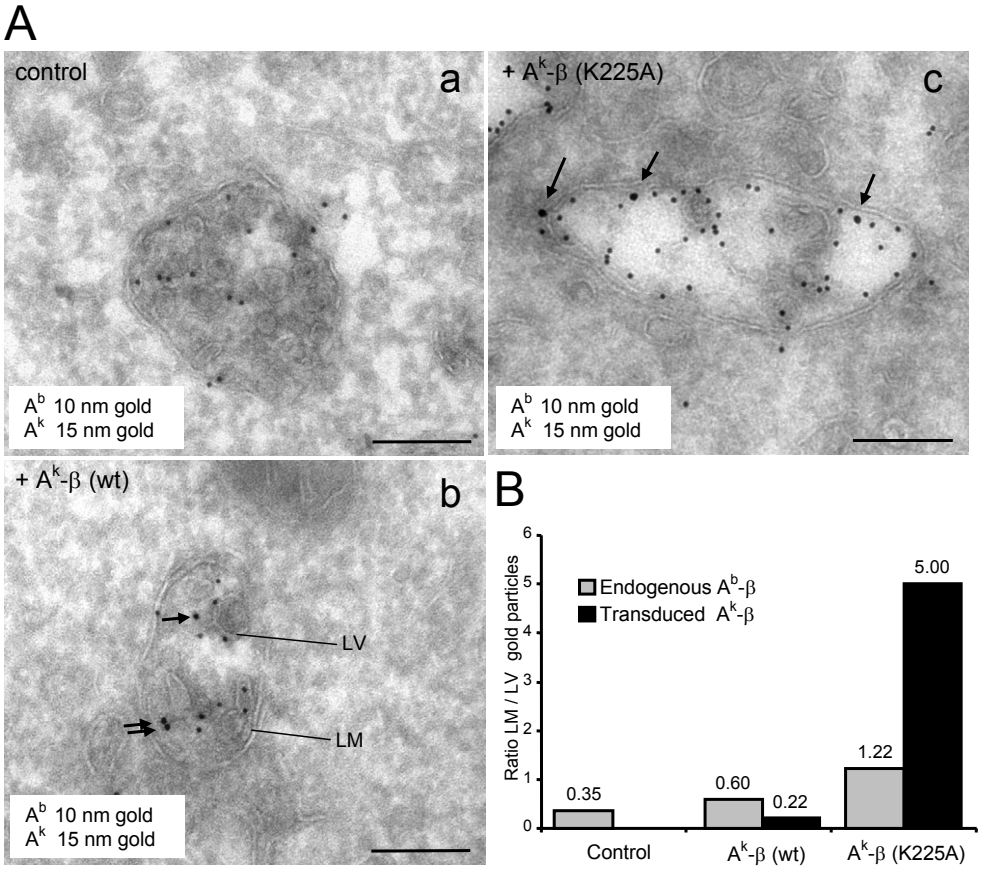

Figure 5. K225 at MHC II-β is required for sorting of MHC II to LV in immature DCs
(A) Ultrathin cryosections of either nontransduced (control) or A^k-β or A^k-β^{K225A}-transduced D1 were double immunogold-labeled for endogenous A^b-β (10 nm gold particles) and A^k-β (15 nm gold particles; indicated by arrows). Examples of MVB are shown with LV and LM indicated. Scale bars represent 200 nm. **(B)** Gold particles on the LM versus LV of MVB were counted, and the ratios (LM/LV) of the totals from >50 MVB profiles for each condition are plotted.

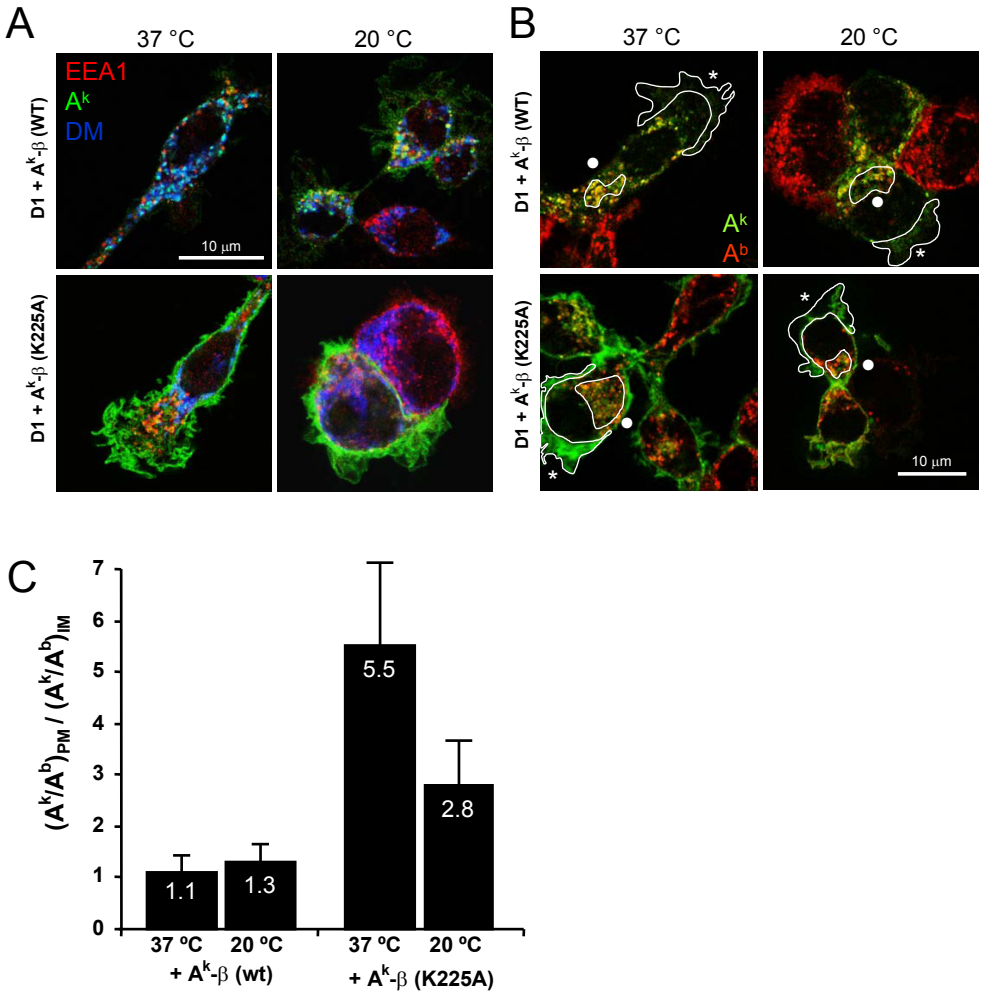

Figure 6. K225 at MHC II-β is required for efficient internalization of MHC II from the plasma membrane

D1 cells were transduced for either A^k-β^{WT} or A^k-β^{K225A}. **(A)** Cells were allowed to bind and endocytose PE-conjugated 10-3.6 for transduced A^k-β (green) at indicated temperatures, fixed, and costained for the early endosomal marker EEA1 (red) and DM (blue). The figure is representative for three independent experiments. **(B)** Cells were allowed to bind and endocytose PE-conjugated 10.3-6 for transduced A^k-β (green) and APC-conjugated M5-114 (red) for endogenous A^b-β for 1 hr at 37 °C or 20 °C as indicated. Cells were washed and fixed, and images were acquired by CSLM. Areas representative for plasma membrane (asterisk) or intracellular structures (dot) were encircled (see indicated examples), and the integrated densities of fluorescence within these areas was determined for 10 distinct samples for each condition. **(C)** The relative efficiencies of uptake were then determined by comparing the ratio of fluorescence at the plasma membrane (PE/APC) with that at the internal membranes (SD are indicated in the bar diagrams and are given in the text).

the plasma membrane ($(A^k/A^b)_{PM}$) with that at intracellular membranes ($(A^k/A^b)_{IM}$) (Figure 6C). A^k-β was endocytosed with a similar efficiency as endogenous A^b-β (ratio 1.1 ± 0.24). In contrast, much less A^k-β^{K225A} was detected intracellularly as compared to labeled A^b-β (ratio 5.5 ± 1.4). This may be explained either by a reduced rate of uptake or accelerated recycling of A^k-β^{K225A}. The latter explanation is plausible considering that lack of sorting at MVB of endocytosed mutant MHC II might well result in accelerated recycling to the plasma membrane. To distinguish between these possibilities, antibodies were endocytosed at 20 ºC, a temperature at which trafficking of endocytosed MHC II to late endosomes and recycling to the plasma membrane are severely hampered [22]. Indeed, after 1 hr uptake at 20 ºC, most endocytosed antibodies reached EEA1-positive early endosomes rather than DM-positive MIIC (Figure 6A). Again, wild-type A^k was taken up as efficiently as endogenous A^b (ratio 1.3 ± 0.25). At this temperature, uptake of A^k-β^{K225A} was less than 50% efficient compared to endogenous A^b-β (ratio 2.8 ± 0.77; Figures 6B and 6C). Thus, ubiquitination of MHC II-β is not only required for sorting at MVB but also for efficient endocytosis. Together, these processes are responsible for intracellular retention of MHC II in immature DCs.

Discussion

We here demonstrate that in immature DCs a unique and conserved lysine residue within the cytoplasmic domain of MHC II-β is oligoubiquitinated after proteolytic processing of MHC II-associated Ii. Ubiquitination of mature MHC II is required both for efficient uptake from the plasma membrane and sorting at MVB into LV. Together, these two sorting steps result in efficient intracellular retention of peptide-loaded MHC II, thereby preventing premature antigen presentation by immature DCs. In mature DCs, increased expression of MHC II-peptide complexes at the plasma membrane has been attributed to several processes: (1) increased transcription of MHC II, (2) increased Ii processing and MHC II peptide loading [23, 24], and (3) extended half life of MHC II resulting from decreased endocytosis rates [25, 26] and recruitment of MHC II from MIIC toward the plasma membrane [2, 20, 27]. We here demonstrate that the recruitment of peptide-loaded MHC II to the plasma membrane can be achieved by blocking

MHC II-β ubiquitination.

Endocytosis of mature MHC II occurs via clathrin-mediated endocytosis [25, 28, 29], and a conserved dileucine-based signal in the COOH terminus of the cytoplasmic domain of MHC II-β is implicated [30]. Elimination of this signal reduced the uptake rate but did not block endocytosis of MHC II. This is consistent with a second endocytosis signal for mature MHC II, which we here identified as an oligoubiquitin tag. Conversely, continued but reduced uptake of MHC II-β^{K225A} in our study may be explained by the dileucine endocytosis motif. In general it is thought that monoubiquitination rather than polyubiquitination is required to downregulate cell-surface receptors by delivering these proteins for lysosome-mediated degradation through the endocytic pathway [7]. However, it has recently been demonstrated that recruitment by tandem ubiquitin-interacting motifs in clathrin adaptors is much more efficient for oligoubiquitinated than for monoubiquitinated membrane proteins [8, 9]. In addition to peptide-loaded MHC II, DCs may also express Ii-associated MHC II [25, 28] (and references therein) and empty MHC II [29] (and references therein) at their plasma membrane. We here demonstrate that Ii-associated MHC II is not ubiquitinated, explaining why surface-exposed MHC II-Ii complexes are endocytosed predominantly through the interaction of two leucine-based signals encoded on the cytoplasmic domain of Ii with the clathrin adaptor AP-2 [25, 28] (and references therein). Surface-exposed empty MHC II lacks Ii-encoded internalization signals but can be endocytosed via the dileucine motif on MHC II-β. Although we show that almost all ubiquitinated MHC II was peptide loaded, we cannot exclude the possibility that also empty MHC II can be ubiquitinated, thereby facilitating endocytosis of empty MHC II.

Monoubiquitination of membrane proteins has particularly been implicated in sorting at MVB [10]. Our finding that oligoubiquitination can serve as a sorting signal for MHC II-β at MVB is, however, not unprecedented, as illustrated by oligoubiquitination of MHC I in Kaposi's sarcoma-associated herpes virus-infected cells, which results in efficient endocytosis and endolysosomal degradation [31]. After uptake of ubiquitinated cargo, sorting at MVB depends on the function of a group of conserved proteins that were originally identified in Saccharomyces cerevisiae (reviewed in [7, 10, 32]). These include Hrs/Vps27 and the constituents of three heteromeric protein complexes called ESCRT-I, II, and III

that act sequentially in sorting at MVB.

Consistent with the observation that endogenous MHC II is ubiquitinated after peptide loading, we observed that also MHC II-β^{K225A} is capable of forming SDS-stable MHC II-peptide complexes (data not shown). This is supported by the observation that B cells expressing MHC II-β^{K225A} were capable of presenting antigens to T cells [33] and is also in concordance with our observation that ubiquitination of MHC II is diminished in response to LPS treatment.

Our finding that LPS and other maturation stimuli (data not shown) rapidly reduce ubiquitination of MHC II suggests strict regulation of this modification and can explain the stable expression and increased half-life of MHC II in maturing DCs. Ubiquitination of MHC II-β in DCs has not been observed previously, which can be explained by the relative small amount of ubiquitinated MHC II-β at steady-state conditions. We favor the idea that cycles of ubiquitination/deubiquitination may occur both at the plasma membrane and endosomes, reminiscent of membrane proteins whose surface expression is regulated either by mono- or polyubiquitination, including several ion channels, tyrosine kinase receptors, cytokine receptors, and T cell and IgE receptors.

Ideally, we would have liked to study whether forced ubiquitination of MHC class II in mature DCs would rescue its endocytosis and sorting at MVB. However, forced expression of ubiquitin ligases or ubiquitin chimeras may target membrane proteins into the MVB pathway, irrespective of the physiological relevance. For example, we considered studying the behavior of transduced MHC II-β ubiquitin chimeric complexes. In our opinion, however, permanent C-terminal monoubiquitin-tagged MHC II-β can be expected to behave differently from transient lysine oligoubiquitin-tagged MHC II-β. First, as indicated above, monoubiquitinated proteins behave differently compared to oligoubiquitinated proteins with respect to endocytosis and MVB sorting. Second, deubiquitination of membrane proteins is crucial for sorting at MVB [34]. Fusing a tag to the MHC II tail would also bypass the elegant switch of sorting motifs that we disclose in this paper. The endosomal sorting motif present in the Ii is removed in endosomes but is replaced by the ubiquitin tag (in concert with the double LL motif in the MHC II-β tail) that drives two distinct sorting events, at the plasma membrane and at the MVB LM. Finally, chimeric ubiquitin tagging of membrane proteins that normally recycle, such as the transferrin receptor, results in accelerated MVB sorting and lysosomal degradation irrespective

of physiological function [35]. An alternative approach would be to force the expression of a ubiquitin ligase. However, as illustrated in a recent paper [36], MHC II can be downregulated when expressed in 293 cells together with several E3-ligases, c-MIR, or MARCH-I, highlighting the promiscuous behavior of these enzymes. Apparently, this system can be pushed with E3 ligases irrespective of their physiologically relevance. Clearly, to characterize the molecular mechanism regulating ubiquitination of MHC class II requires more subtle approaches, and the complexity of the mechanism may resemble that for other receptors, such as for receptor tyrosine kinases [37].

Our observation that ubiquitination of MHC II-β requires prior processing of Ii is consistent with the finding that DCs from cathepsin S-deficient mice showed aberrant Ii processing and MHC II trafficking [38, 39]. In this respect, it is interesting to note that nonameric MHC II-αβ Ii complexes dissociate into dimeric MHC II-αβ complexes as a result of processing of p10 into CLIP. Possibly, association with Ii prevents premature exposure of MHC II-β to the ubiquitination machinery. Our finding that MHC II-β is ubiquitinated after Ii processing in DCs at physiological conditions extends on the observations by Ohmura-Hoshino et al., 2006, and suggests that an endogenous ligase, possibly a member of the MARCH family, may associate only after liberation of MHC II-αβ dimers in response to Ii processing. Ubiquitination of MHC II-β is, however, likely to be regulated by factors other than E3 ligase activity because ubiquitination was strongly reduced already after 1-2 hr of LPS treatment, a condition at which Ii processing was rather accelerated (Figure 3B and data not shown). In our opinion, the machinery responsible for regulating MHC class II ubiquitination can be identified by functional interference only. This complex task is subject to current research.

In conclusion, the described molecular mechanism for regulating surface expression of MHC II is essential for DCs to modulate antigen presentation in response to pathogens. It can be envisioned that artificial interference with MHC II ubiquitination can be a powerful approach to modulate the immune system for treatment of cancer, vaccination, or treatment of autoimmune disease.

Materials and Methods

Reagents

D1, an immature DC line from C57Bl/6 mice, was cultured as described [11]. BMDCs were isolated as described [40] from the bone marrow of WT C57BL/6 mice or B6.129-H2-Ab1^{tm1Gru} N12 (Taconic, Denmark), an A^b-β-deficient C57BL/6 mouse [41].

LPS (Escherichia coli, serotype 026:B6) was used at 10 µg/ml, Leupeptin at 100 µg/ml, MG132 at 20 µM, and E64d at 100 µM (all from Sigma). Rabbit polyclonal antibodies directed against the cytoplasmic domains of DM [42], MHC II-β, and Ii [43] were from N. Barois (University of Oslo, Oslo, Norway). Anti-mouse MHC II M5/114 [44] and Y3P [45] were both provided by S. Amigorena, and 15G4, directed against A^b/CLIP [39], was a gift of A. Rudensky. HRP-conjugated and agarose bead-conjugated anti-ubiquitin P4D1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rat anti-mouse isotype control was purchased from Serotec (Raleigh, SC), and anti-tubulin 1A2 was from Sigma.

Protein Isolation and Analyses

For immunoprecipitations, DCs were lysed at 4 °C in 1% Triton X100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl (pH 8), 10 mM N-ethylmaleimide (Sigma), and complete protease inhibitor mix (Roche Molecular Biochemicals, Almere, The Netherlands), and nuclei were removed by centrifugation. Immunoprecipitations were performed as indicated in the figure legends. Protein G-agarose beads (Sigma Aldrich, Zwijndrecht, The Netherands) were precoupled to either Y3P, M5/114, 15G4, or IgG2b rat anti-mouse isotype control antibodies in lysis buffer, added to the cell lysates, and gently mixed for 1 hr at 4 °C. The beads were then washed, and immunoprecipitated MHC II was eluted either in SDS sample buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.8], 10% Glycerol) for 30 min or 16 hr at room temperature or 5 min at 100 °C. When indicated, MHC II was eluted in 1% SDS at 100 °C for subsequent immunoprecipitation of ubiquitinated proteins. For this goal, the eluate was diluted 10 times in lysis buffer and precleared for nonspecific binding with nonrelevant IgG2-conjugated agarose beads. Supernatants were collected and incubated with anti-ubiquitin P4D1

conjugated agarose beads or control antibody-conjugated beads, both from Santa Cruz Biotechnology for 1 hr at 4 ºC. Nonspecifically associated proteins were removed by washing in 4 M Urea, after which ubiquitinated proteins were eluted off the beads by boiling in SDS sample buffer. Western blotting was performed according to standard techniques. For mass spectrometric analyses, protein bands were subjected to in-gel tryptic digestion and subsequent LC-MS/MS analysis as described previously [46]. The data files obtained from LC-MS/MS experiments were converted into peaklist files (.pkl files) with ProteinLynx Browser version 2.1 software (Micromass) and subsequently submitted to the Mascot search software (Matrix Science, London, UK) [47]. The allowed peptide mass tolerance was 0.4 Da, fragment mass tolerance of 0.2 Da, allowing 2 missed cleavages, to search the mouse IPI database version 3.11. [48].

Constructs and Retroviral Transduction of DCs

A^k-β^{WT} and A^k-β^{K225A} cDNA in pRc/CMV vector were kindly provided by T. Laufer (Harvard School of Public Health, Boston, MA) [33]. cDNAs were amplified by PCR and cloned into pQCXIN (Clontech, Mountain View, USA), and sequences were verified. Retroviral particles were produced by 293T Phoenix-Eco cells after cotransfection with the pQ vectors and pCl-Eco (provided by H. Rozemuller, Utrecht Medical Centre, Utrecht). Harvested viruses were filtered, mixed with polybrene (4 µg/ml, Sigma), and used to infect either D1 or BMDC by spin occulation 7 days after isolation. Transduced BMDC were analyzed after 2 days of expression by immunofluorescence or FACS, and transduced D1 were maintained as stable cell cultures.

Microscopy

Cells grown on glass coverslips were fixed and immunolabeled for DM (2E5A, BD Biosciences, San Jose, CA), for A^b-α (Y3P), for A^k-β (PE-10.3-6, BD Biosciences or 10.2.16, kind gift of S. Ostrand-Rosenberg). Nonlabeled antibodies were visualized with donkey anti-mouse Alexa 568 or goat anti-rat Alexa 488 (Molecular Probes, Eugene, OR). Confocal microscopy was performed on a Bio-Rad Radiance 2100MP confocal and multiphoton system (Zeiss/Bio-Rad, Hertfordshire, UK).

For internalization studies, labeled antibodies (PE 10.3-6 and APC M5-114,

both from BD Biosciences) were incubated with transduced D1 for 1 hr at indicated temperatures. Cells were washed and fixed, and images were acquired by confocal microscopy. Relative presence of both antibodies was measured as integrated densities (ID) from regions of interest drawn either inside cells or on the plasma membrane of confocal sections. Ratios (plasma membrane divided by the internal pool ID) of these values were determined for each marker in 10 cell profiles for each condition with Image J software.

For immunoelectron microscopy, cells were fixed, and single- or double-immunogold labelings of ultrathin cryosections were performed as described [49]. Sections were observed under a Philips CM120 Electron Microscope (FEI Company, Eindoven, The Netherlands) equipped with a digital camera Keen View (SIS, Germany). A^b-β was labeled with M5/114, followed by rabbit anti-rat Ig (Dako, Denmark) and protein A conjugated to 10 nm gold particles. A^k-β was labeled with mouse monoclonal 10.3-6 followed by protein A conjugated to 15 nm gold particles. Protein A conjugated to gold particles were purchased from Cell Microscopy Centre (AZU Utrecht, the Netherlands). Label for MHC II was considered to be associated with the LM when the gold particle was within 20 nm of the LM and with no cross-sectioned LM in the direct vicinity.

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Degradation but not peptide loading of MHC class II in dendritic cells requires VPS4 driven sorting at multivesicular bodies

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To be submitted

Abstract

Dendritic cells (DCs) present peptides in association with major histocompatibility complex class II (MHCII) to naïve CD4$^+$ T cells to initiate adaptive immune responses. Loading of peptide antigens from exogenously acquired material onto MHCII occurs within the endocytic tract. From there, peptide loaded MHCII can either be transported to the plasma membrane or transferred to lysosomes for degradation. The latter pathway is dominant in immature DCs and requires ubiquitination of MHCII for sorting into intraluminal vesicles (ILVs) of multivesicular bodies (MVBs). Whether ILVs can also serve as a site for MHCII peptide loading and than fuse back with the endosomal delimiting membrane is debated. We here investigated the dependence of MHCII processing on VPS4, an ATPase that is essential for both finalizing ESCRT driven sorting of ubiquitinated cargo at MVBs and ILV formation. Overexpression of an EGFP tagged dominant-negative ATPase-deficient mutant of VPS4B, EGFP-VPS4B^{E235Q}, did not affect shuttling of MHCII between the plasma membrane and endosomes but significantly prolonged its lifetime, consistent with a role in sorting of MHCII at MVBs for lysosomal degradation. The stability of MHCII complexes in sodium dodecyl sulphate was also not affected, indicating that peptide loading does not require passage of MHCII through ILVs.

Introduction

The initiation of adaptive immune responses requires activation of naive CD4$^+$ T cells by dendritic cells (DCs) through presentation of antigenic peptides by major histocompatibility class II (MHCII) molecules [1]. This process is highly controlled, and one level of regulation is achieved through intracellular trafficking of MHCII [2]. Immature DCs are distributed throughout peripheral tissues where they survey their environment for the presence of conserved pathogen associated molecular patterns (PAMPs). Recognition of PAMPs by specific receptors initiates a maturation program that increases the efficiency of antigen presentation to T

cells. Already at a non-activated state DCs express MHCII, which is constitutively loaded with peptides derived from self antigen [3-5] (and references therein). These molecules may be important for antigen presentation by tolerogenic immature DCs [6]. The display of peptide loaded MHCII (pMHCII) at the plasma membrane of non-activated DCs is however limited as a consequence of its sorting at endosomes for lysosomal degradation. Sorting of MHCII to lysosomes is driven by ubiquitination of a highly conserved single lysine residue within the cytoplasmic domain of its MHCII-β chain [7, 8]. Ubiquitination of MHCII in DCs is driven by MARCH I [9, 10], and possibly to a minor extend also by other E3 ligases such as MARCH VIII [9, 11]. Of note, ubiquitination of MHCII requires prior degradation of the invariant chain [7], the MHCII associated chaperone that directs newly synthesized MHCII to the endocytic pathway [12]. Peptide loading of MHCII was found to occur already before its ubiquitination [7]. After peptide loading in endosomes, MHCII may either be transferred to the plasma membrane or directed to lysosomes for degradation. When pMHCII is transported to the cell surface, it can be endocytosed and in second instance be sorted to lysosomes for degradation. We and others have shown that ubiquitination of pMHCII may facilitate its endocytosis [7, 8], although the mechanisms for endocytosis of pMHCII are probably multifactorial and poorly understood. In addition to the ubiquitin moiety, also a conserved di-leucine motif in the cytosolic domain of the β-chain [13, 14], incorporation into protein/lipid domains [15], as well as a clathrin-independent endocytosis pathway [16] have been implicated in the endocytosis of pMHCII.

In immature DCs, MHCII is sorted for lysosomal degradation within the endocytic pathway through incorporation into the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs). Efficient sorting of MHCII to ILVs requires its ubiquitination [7, 8] and is probably driven by the endosomal sorting complex required for transport (ESCRT) [2]. Antigen presentation is promoted after activation of conventional DCs, but not in plasmacytoid DCs [17], by increasing the stability of pMHCII [2, 17]. Stabilization of MHCII in response to an activation signal is a direct consequence of interference with its ubiquitination [7, 8]. The molecular mechanism for attenuation of MHCII ubiquitination is unclear but may either occur through down-regulation of MARCH I (activity) [10, 18], or by increasing MHCII deubiquitination. Enzyme(s) for MHCII deubiquitination have yet to be identified. As an alternative mechanism in activated DC, CD83

was shown to sequester MARCH I away from MHCII [19]. Possibly other, yet undiscovered mechanisms may also reduce MHCII ubiquitination. Conversely, the anti-inflammatory cytokine IL-10 was shown to increase MARCH I expression [20], thus promoting MHCII degradation at immune suppressive conditions.

At the endosomal system, sorting of K63-linked oligoubiquitinated membrane proteins and formation of ILVs is facilitated by the endosomal sorting complex required for transport (ESCRT) [21, 22]. The ESCRT machinery is comprised of at least four distinct complexes, ESCRT-0, -I, -II, and -III. ESCRT-0 consists of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducer adaptor molecule) subunits, both of which are able to bind ubiquitin, allowing ESCRT-0 to recruit ubiquitinated membrane proteins at the endosomal surface. Hrs also binds clathrin via its clathrin box motif allowing packaging of ubiquitinated cargo into flat clathrin lattices at the endosomal delimiting membrane. These clathrin lattices can be observed by electron microscopy as a characteristic flat electron dense coat at the cytoplasmic face of the endosome [23, 24] and have also been demonstrated to concentrate MHCII [2]. ESCRT-I also binds ubiquitinated cargo and ESCRT-0 while ESCRT-II in its turn binds to ESCRT-I and drives the assembly of ESCRT-III which promotes inward budding of the endosomal membrane. At this point ubiquitin is recycled from the sorted molecules by the action of deubiquitinating enzymes such as AMSH (associated molecular with SH3 domain of STAM), and UBPY (ubiquitin peptidase Y). The final step involves the vacuolar protein sorting complex Vps4/SKD1, an ATPase that is required to dissociate ESCRT-III from the membrane and for the actual fission of ILVs [25]. Recently it was demonstrated in yeast that the presence of ubiquitinated substrates for ESCRT at the endosomal delimiting membrane is essential for the formation of ILVs [26]. Mammalian cells that are depleted for key ESCRT components are however still able to generate ILVs [27], possibly as a consequence of compensating ESCRT components or the involvement of certain lipid domains that also facilitate ILV formation [28]. For example, depletion of ESCRT-II by small interfering RNA did not affect degradation of activated epidermal growth factor [29], another cargo of the multivesicular body protein sorting pathway. Similarly, down-regulation of major histocompatibility complex class I in cells expressing the Kaposi sarcoma-associated herpes virus protein K3 was unaffected in ESCRT-II depleted cells [29]. Collectively, such data suggest that some

ESCRT components may be redundant or that homologous subunits compose distinct ESCRT complexes that act on specific cargoes [30]. Another complication for studying the role of the MVB sorting machinery in MHCII trafficking is that quantitative depletion of ESCRTs in DCs by small interfering RNA or introduction of dominant-negative constructs is notoriously difficult and eventually lethal. To determine whether sorting of MHCII to lysosomes is dependent on ESCRT driven mechanisms, we performed experiments on sorted DCs that expressed EGFP tagged VPS4B^{E235Q} just two days after retroviral transduction. VPS4 is essential for finalizing all ESCRT catalyzed sorting and expression of VPS4B^{E235Q}, an ATPase deficient dominant-negative mutant of VPS4, is generally used as a tool to detect ESCRT-dependence [21]. Indeed, overexpression of VPS4B^{E235Q} in mammalian cells strongly reduced the formation of ILVs [31]. We here show that VPS4B^{E235Q} expression increased the lifetime of MHCII in immature DCs but did not interfere with MHCII peptide loading, consistent with the idea that incorporation into ILVs is a prelude to lysosomal degradation but not required for MHCII peptide loading.

Results

DCs cells were transduced with a retroviral expression system to express either EGFP tagged wild type VPS4B or an EGFP tagged ATPase-deficient mutant of VPS4B, EGFP-VPS4B^{E235Q}. All experiments were performed 45-48 hrs after transduction. Longer expression of EGFP-VPS4B^{E235Q} had adverse effects on cell viability (data not shown). Both constructs were expressed in 5-15% of the cells. EGFP-VPS4B was distributed throughout the cell while EGFP-VPS4B^{E235Q} was concentrated at but also near to enlarged intracellular MHCII containing compartments (Figure 1). This is consistent with the role of VPS4 in dissociating ESCRT from late endosomal compartments [36, 37] and with other studies that reported on the accumulation of EGFP-VPS4B^{E235Q} on late endosomal compartments in other cell types [31, 33]. Of note, EGFP-VPS4B^{E235Q} also localized at or near to compartments containing DM, which at control conditions and in contrast to MHCII, is largely present at the endosomal delimiting membrane rather than at ILVs [38], again consistent with its accumulation at late endosomes.

From these images it already became apparent that in EGFP-VPS4B^{E235Q} expressing cells, MHCII was more prominently present on the plasma membrane in comparison to EGFP-VPS4B expressing cells (Figure 1). This characteristic phenotype was clearly observed for 58% of EGFP-VPS4B^{E235Q} expressing cells (n=97 from 11 independent experiments). To quantitatively compare surface expression of MHCII at distinct conditions, we performed flow cytometry on transduced cells. Hereto, cells were first stimulated for 16 hrs with LPS or left unstimulated, then fixed, and labelled with fluorescent antibodies for MHCII without cell permeabilization. MHCII surface expression was determined for both EGFP-negative cells and EGFP gated cells from the same cultures (Figure 2). MHCII cell surface expression was 2,1 ± 0,4 fold (n=4, $p<0,05$) increased in EGFP-VPS4B^{E235Q} expressing immature DCs but not in EGFP-VPS4B expressing immature DCs. In comparison, 16 hrs stimulation by LPS increased cell surface expression of MHCII 2,6 ± 0,6 fold (n=4 $p<0,05$). DCs were not activated by EGFP-VPS4B or EGFP-VPS4B^{E235Q} as determined by the surface expression

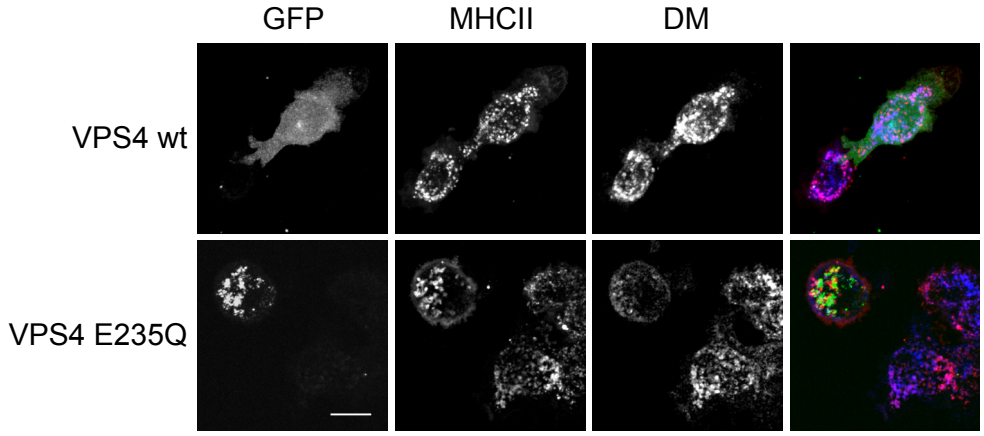

Figure 1. EGFP-VPS4B^{E235Q} localizes to intracellular MHCII containing compartments and increases MHCII at the plasma membrane
Cells were transduced for EGFP-VPS4B or EGFP-VPS4B^{E235Q} (green) and immuno-labelled for MHCII (red) and DM (blue). Individual labels for the same cells are presented in black and white as indicated, merged pictures are in colour. In expressing cells, EGFP-VPS4B is distributed throughout the cytosol while EGFP-VPS4B^{E235Q} is predominantly present on intracellular compartments that largely overlap with those containing MHCII and/or DM. Note that MHCII label on the plasma membrane of the EGFP-VPS4B^{E235Q} expressing cell is higher in comparison to that on EGFP-VPS4B^{E235Q} negative cells. Scale bar, 5 µm.

of CD86, a co-stimulatory molecule that is highly up-regulated in activated DCs. Indeed after 16 hrs LPS surface CD86 was up 9,0 ± 1,9 fold (n=4, p<0,05) while EGFP-VPS4B^{E235Q} expressing non-stimulated cells displayed a 2,2 ± 0,2 fold increase. CD86 is not only transcriptionally regulated but, like MHCII, also differentially sorted at MVB depending on the activation status of the DC [39, 40]. Interference with this pathway probably explains the relative mild increase of surface CD86 in EGFP-VPS4B^{E235Q} expressing cells in comparison to LPS activated DCs. We observed that the surface expression of MHCII of individual cells increased proportional to expression levels of EGFP-VPS4B^{E235Q} (Figure 2 C,D). Cells with maximal expression of EGFP-VPS4B^{E235Q} displayed > 3 fold increase in surface expression of MHCII. The effect of EGFP-VPS4B^{E235Q} on MHCII surface expression could result either from interference with trafficking between the plasma membrane and endosomes or be a consequence of intervention with lysosomal targeting and degradation, resulting in higher total cellular levels of MHCII (Figure 3A). To determine whether the shuttling of MHCII between the plasma membrane and intracellular compartments was affected we compared cell surface MHCII with total cellular MHCII. Total cellular MHCII was determined by labelling after cell permeabilization with saponin. Like surface exposed MHCII, total MHCII was increased by the expression of EGFP-VPS4B^{E235Q} but not of EGFP-VPS4B (Figure 3B,C). Intriguingly, the distribution of MHCII on plasma membrane relative to intracellular compartments was completely unaffected by EGFP-VPS4B^{E235Q} (Figure 3D). This indicates that shuttling of MHCII between the plasma membrane and endosomes was not disturbed by EGFP-VPS4B^{E235Q}. We conclude that the overall increase in MHCII was due to aborted endosomal sorting for lysosomal degradation (Figure 3A).

Having established that EGFP-VPS4B^{E235Q} interfered with sorting of MHCII in endosomes we next asked whether MHCII peptide loading was affected. Hereto, 10^5 cells of each EGFP-VPS4B and EGFP-VPS4B^{E235Q} expressing cells were sorted from non-expressing cells by flow cytometry, lysed in SDS sample buffer at room temperature, and analysed by immonoblotting for MHCII-β chain (Figure 4). Only peptide loaded MHCII-αβ dimers are stable in SDS and these can easily be separated by SDS-PAGE from free MHCII-β chains and from non-peptide loaded MHCII. Consistent with the flow cytometric data, total MHCII increased about two fold in EGFP-VPS4B^{E235Q} expressing cells. Notably, the ratio of SDS

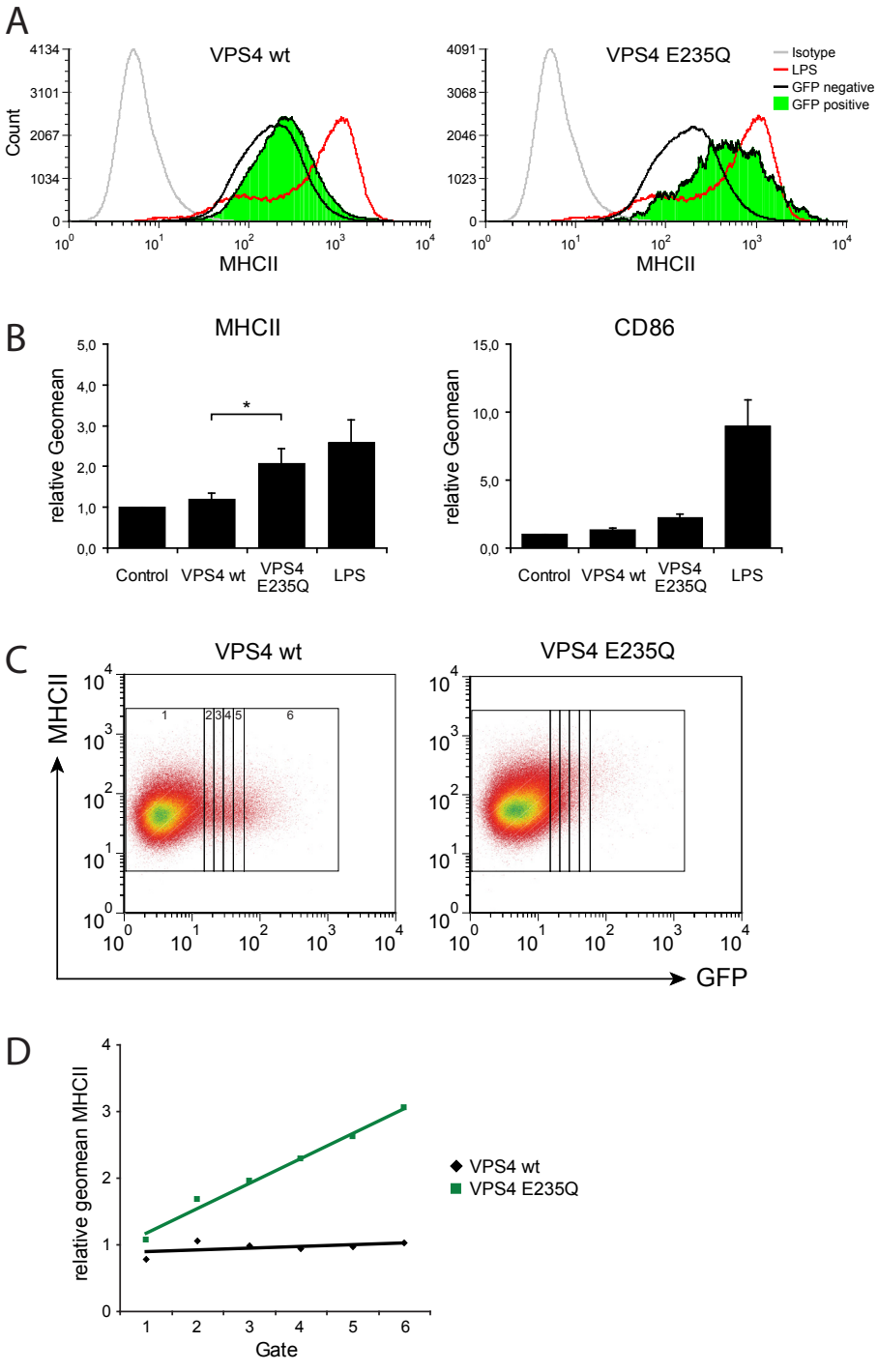
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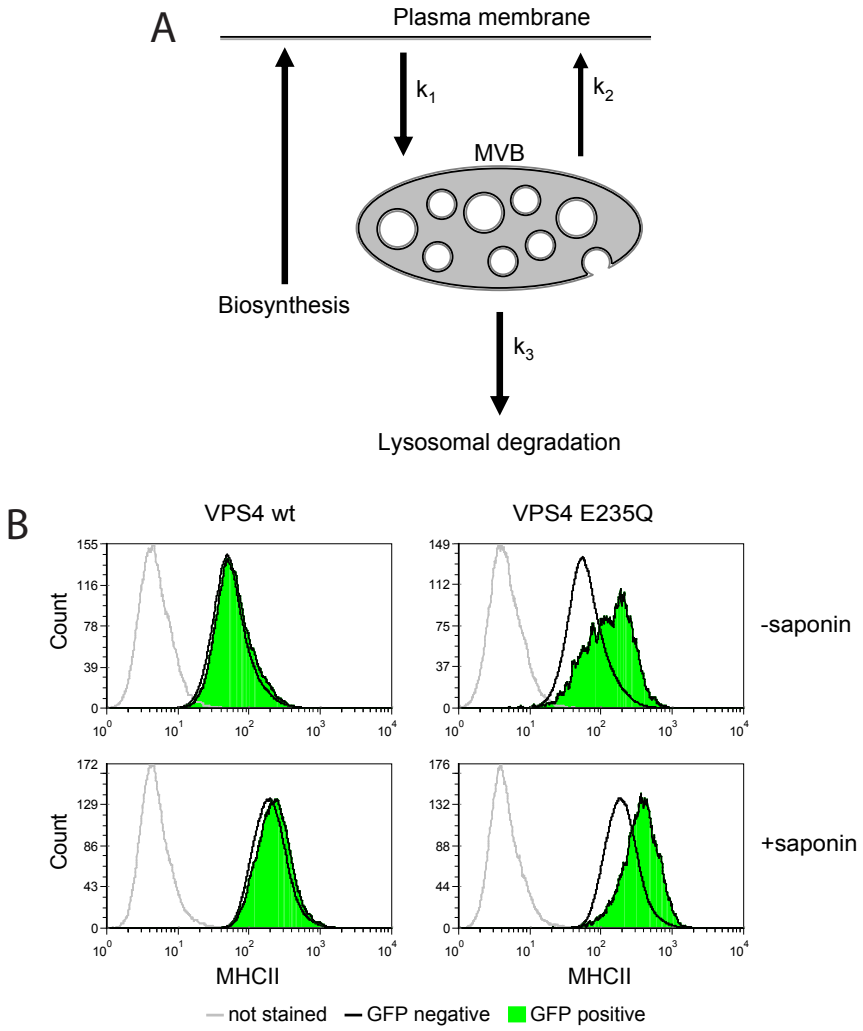
Figure 2 (left). EGFP-VPS4B^{E235Q} increased cell surface expression of MHCII
(A) Cells were transduced for EGFP-VPS4B or EGFP-VPS4B^{E235Q} or stimulated with LPS. EGFP positive and negative cells were gated separately and analysed for MHCII surface expression by flow cytometry. Background labelling was assessed using an isotype control antibody. **(B)** Left panel, geomeans for MHCII expression above background as exemplified in figure 2A are plotted as relative values in comparison to that of EGFP-negative cells (± SD from 4 independent experiments). Expression of MHCII by LPS treated cells was determined for EGFP negative cells. * Significant difference with p = 0,002. Right panel, relative expression levels of CD86 were determined as for MHCII as a measure for DC activation. **(C)** Cell surface expression of MHCII increased proportional with EGFP-VPS4B^{E235Q} expression. Primary plots of MHCII expression by EGFP-VPS4B and EGFP-VPS4B^{E235Q} transduced cells with gate 1 for EGFP negative cells and gates 2 to 6 for increasing EGFP expression. **(D)** Relative geomeans of MHCII expression in gate 1-6 as set in figure 3C. Figures are representative of 4 independent experiments.

stable versus unstable MHCII was unaffected by EGFP-VPS4B^{E235Q}, indicating that the peptide loading process was not obstructed at these conditions. We conclude that VPS4 driven sorting of MHCII into ILVs is not required for peptide loading.

Discussion

Expression of MHCII at the DC plasma membrane is determined by its synthesis, the dynamics of internalization, intracellular retention and degradation. Cell surface expression increases during DC maturation due to modulation of all of these parameters. MHCII synthesis is temporally increased in response to maturation signals, but virtually absent in fully matured DCs [4, 5, 41-45]. The fate of the MHCII cohort that is synthesized during DC maturation is different from the majority of MHCII that was synthesized prior to DC activation as a consequence of changes in its trafficking. In immature DCs, approximately 60% of total cellular MHC class II is located at MVBs, and of this population ~80% resides at ILVs and the remainder at the delimiting membrane. The population at ILVs is prone to be transferred to lysosomes for degradation. MHCII that was mutated at its unique ubiquitination site was inefficiently incorporated into ILVs of immature DCs [7, 8]. MHCII that is not incorporated into ILVs can instead be transferred to the plasma membrane. It was noted however that also endocytosis of pMHCII was dependent on ubiquitination, albeit to a lesser extent [7, 8, 46]. It is thus debated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Plasma membrane
k1
k2
MVB
Biosynthesis
k3
Lysosomal degradation
B
VPS4 wt
VPS4 E235Q
-saponin
+saponin
Count
MHCII
not stained
GFP negative
GFP positive
C
Total MHCII
relative Geomean
Control
VPS4 wt
VPS4 E235Q
D
MHCII
relative Geomean ratio (PM/total)

Figure 3 (left). EGFP-VPS4B^{E235Q} did not change the relative distribution MHCII between plasma membrane and intracellular compartments
(A) Diagram showing the major intracellular pathways of MHCII. MHCII can be internalized and transported to endosomes with a rate constant k1 and transported further to lysosomes for degradation with a rate constant k3, by making use of the ESCRT machinery that recognizes ubiquitinated MHCII driving it to be incorporated into the ILV of MVB. Alternatively, endocytosed MHCII may recycle from endosomes back to the plasma membrane with a rate constant k2. When EGFP-VPS4B^{E235Q} would interfere with k3 only, total cellular MHCII would increase but the ratio of cell surface versus intracellular MHCII would stay constant. Alternatively, when either k1 or k2 would also be affected, the relative distribution of MHCII could be expected to change accordingly. **(B)** Cells were transduced for EGFP-VPS4B or EGFP-VPS4B^{E235Q}, fixed and immuno-labelled for MHCII in the absence or presence of saponin. Examples of flow cytometry profiles of cells labelled for surface MHCII (- saponin) and total cellular MHCII (+ saponin) are shown. **(C)** Geomeans for total cellular MHCII above background as exemplified in figure 3B are plotted as relative values in comparison to that of EGFP-negative cells (± SD from 3 independent experiments). Geomeans for cell surface MHCII above background are not shown but were similar to those shown in figure 2B. **(D)** Relative ratio of the geomeans of plasma membrane versus total cellular MHCII. The distribution of MHCII was not changed in cells expressing EGFP-VPS4B or EGFP-VPS4B^{E235Q}.

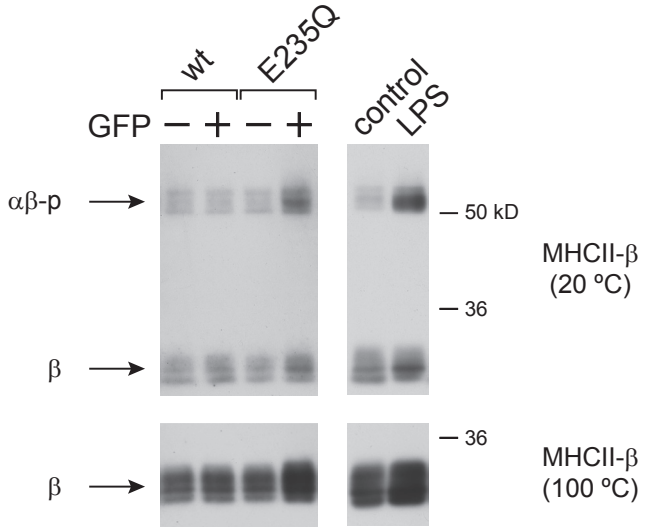

Figure 4. MHCII peptide loading is not affected by EGFP-VPS4B^{E235Q}
Cells were transduced for EGFP-VPS4B or EGFP-VPS4B^{E235Q} and sorted for the expression of EGFP. 10^5 of each EGFP – and EGFP + sorted cells were lysed in SDS sample buffer at either 20 °C (upper panels) or 100 °C (lower panels) and analysed by Western blotting for MHCII-β. Although the total amount of MHCII-β was increased in EGFP-VPS4B^{E235Q} expressing cells, the ratio of peptide loaded MHCII (αβ-p), as indicated by its resistance to dissociation in SDS at 20 °C, was unchanged.

which sorting step, at the plasma membrane or at MVB, is the most important determinant for MHCII surface expression. We here demonstrate that the total amount of MHCII in immature DCs was increased by EGFP-VPS4B^{E235Q} expression. VPS4 has a major role at a late stage of MVB sorting, it dissociates ESCRT-III from endosomes and is required for the actual scission of ILVs [22]. Cells expressing EGFP-VPS4B^{E235Q} have indeed been demonstrated to generate endosomes containing relatively few ILVs [31]. VPS4 has no documented role in regulating endocytic uptake. Ligand activated receptor tyrosine kinases can be polyubiquitinated and undergo ESCRT-mediated sorting to ILVs, resulting in their degradation in lysosomes [47]. Epidermal growth factor receptor is actively recruited into ILVs and degraded in lysosomes in a VPS4 dependent manner [48]. Analogously, we conclude that sorting of MHCII at MVBs is a major regulatory step for MHCII surface expression, but do not exclude additional regulation through endocytosis. It has however also been reported that transferrin receptor, low-density lipoprotein receptor, and asialoglycoprotein receptors were depleted from the plasma membrane of VPS4B^{E235Q} expressing cells as a consequence of interference with their recycling after endocytosis [48, 49]. We did not observe a redistribution from the plasma membrane to intracellular compartments of MHCII in EGFP-VPS4B^{E235Q} expressing DCs (Figure 3D). The discrepancy between these observations remains unresolved but may be explained by the fact that nutrient receptors and MHCII utilise distinct pathways for their transport from endosomes to the plasma membrane. In contrast to early endosomes, from which nutrient receptors recycle by default, late endosomes are not characterised by intense membrane recycling [50]. In activated DCs, characteristic long tubules and possibly vesicular intermediate transport vesicles are formed from late endocytic compartments to transfer MHCII to the plasma membrane [38, 51-54]. Recently, the export pathway of MHCII in immature DCs was demonstrated to be controlled by the motor protein myosin 1E, its receptor ARF7EP and upstream regulator ADP-ribosylation factor-like protein 14 [55]. In view of our results, this pathway appears to be resistant to EGFP-VPS4B^{E235Q}, in contrast to the nutrient receptor recycling pathway.

Exposure of the nonameric MHCII-invariant chain complex to endosomal proteases results in the removal of the Ii-encoded sorting signals from the complex, the release of MHCII-αβ dimers, and subsequent loading of MHCII

with peptides. All these processes occur constitutively and efficient in immature and activated DCs [3] (and references therein). pMHCII can either be sorted to lysosomes for degradation or transported to the plasma membrane, with the former pathway more prominent in immature DCs and the latter prevailing in activated DCs. It has been debated whether peptide loading takes place at the delimiting membrane or at ILVs. DM is an important facilitator for MHCII peptide loading and like MHCII, has been located on both the endosomal delimiting membrane as well as on ILVs [38]. Both sites provide access to antigenic peptides that are generated in the same MVB lumen. In a study using fluorescence resonance energy transfer on transfected HEK293 it was demonstrated that fluorescently tagged MHC class II and DM primarily interacted at ILVs rather than at the delimiting membrane of artificially swollen MVBs [56]. These data suggest that peptide loading of MHC class II, by preference, may happen at this location. When ILVs would indeed be an essential site of peptide loading, a pathway should exist for pMHCII to travel back to the delimiting membrane from where it could reach the plasma membrane. Such a pathway, also referred to as the back or retro-fusion of ILVs, has also been proposed to provide the membrane material necessary for the formation of the MVB-associated tubules that are induced in maturing DCs [38, 51-53]. ILV back-fusion has however never been recorded in live cells. Our current observation that MHCII peptide loading was not affected in EGFP-VPS4B^{E235Q} expressing cells indicates that transfer to ILVs is not required for MHCII peptide loading. This is consistent with previously made observations. Firstly, MHCII failed to sort to ILVs in cathepsin S deficient cells [57, 58]. Indeed ubiquitination of MHCII can occur only after associated invariant chain has been proteolytically removed [7], making MHCII susceptible to peptide loading already when still at the delimiting membrane. Secondly, mutant MHCII that cannot be ubiquitinated, and thus not actively recruited to ILVs, was efficiently loaded with peptide [7, 59]. Thirdly, the majority of ubiquitinated wild type MHCII was already peptide loaded as determined by the stability of the complex in SDS [7]. Collectively, these data imply that, also due to the high rate of DM-driven CLIP-peptide exchange, peptide loading occurs in parallel and even faster than the addition of ubiquitin as the sorting signal for ILVs. Furthermore we have recently demonstrated that activated DCs use newly synthesized MHCII rather than "ILV-stored" MHCII for antigen presentation [4]. Finally, in immature

DCs about 50% of total MHC class II is located at the ILVs [4, 38] and loaded with self-peptides. Recruitment of such a pre-assembled pool of pMHCII in activated DCs would even be undesirable as this would facilitate presentation of self-peptides at inflammatory conditions, possibly evoking autoimmunity. Although highly reduced, some ubiquitination deficient MHCII was still targeted to ILVs, possibly taken along by default membrane transport [7, 8]. From that point of view, the possibility that MHCII peptide loading or editing could additionally occur at ILVs can not be fully excluded. However, our current observation that interference with ILV formation did not affect MHCII peptide loading strongly argues against any essential role of ILVs in MHCII peptide loading.

Materials and Methods

Cell culture

The mouse DC cell line D1 [32], was maintained in Iscove's Modified Dulbecco's medium supplemented with 2 mM Ultraglutamine 1 (both from Biowhittaker), 50 μM β-mercaptoethanol and 30% conditioned medium from GM-CSF producing NIH 3T3 cells (R1) [32]. 293T Phoenix-Eco cells were cultured in DMEM containing 4,5 mg/ml glucose and 2 mM Glutamax (GIBCO). All media were supplemented with 10% heat inactivated FCS (Sigma-Aldrich) and 100 IU/ml penicillin and 100 mg/ml streptomycin (both from GIBCO). For maturation, DCs were incubated with 10 μg/ml LPS (Escherichia coli, serotype 0111:B4 from Fluka) for 18-24 hours.

Constructs and retroviral transduction

Plasmids (pEGFP-C) containing constructs of the enhanced green fluorescent protein (EGFP) fused to the N-terminus of wild type human VPS4B or VPS4B^{E235Q} were kindly provided by Philip Woodman [33]. The EGFP-VPS4B cDNAs were excised and subcloned by BamHI and PinAI in the pQCXIX retroviral vector (Becton Dickinson Biosciences Clontech). 293T Phoenix-Eco cells were allowed to produce retroviral particles for 18–22 hours at 37 °C after $Ca_3(PO_4)_2$ mediated cotransfection of the VPS4 constructs and pCL-Eco (kindly provided by H. Rozemuller, Utrecht Medical Center, Utrecht). Harvested viruses were centrifuged

for 5 minutes at 1000x*g*, filtered by passage through Millipore 0.45 µm filters and used to infect DCs while centrifuging at 1000x*g* for 90 minutes. Virus supernatant was replaced with new medium and cells were cultured for 2 hrs after which the infection procedure was repeated with fresh virus. Transduced DCs were cultured for ~45-48 hrs and then analyzed by immunofluorescence microscopy, flow cytometry and immunoblotting.

Microscopy

DCs grown on glass coverslips were transduced as described, fixed in 0,1 M phosphate buffer pH 7,4 containing 4% paraformaldehyde, and permeabilized for 15 minutes in PBS containing 2% BSA and 0,1% saponin (Riedel de Haën). Cells were blocked and immuno-labelled for H2-M with monoclonal antibody 2E5A (Becton Dickinson Biosciences) and I-A with monoclonal antibody M5/114 (eBiosciences) or Y3P [34] in PBS/2% BSA/0,1% saponin. Monoclonal antibody M5/114 was used to label MHCII in Figure 1. Primary antibodies were labelled with donkey anti-mouse Cy5 (DAKO) and Goat-anti rat Alexa 568 (Invitrogen/Life Sciences). Confocal microscopy was performed on a Bio-Rad Radiance MP2100 confocal and multiphoton system (Zeiss/Bio-Rad, Hemel Hempstead,UK). Images were background subtracted, maximum projections were performed and colour coded using FIJI/ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD).

Flow cytometry

DCs were harvested in PBS containing 2 mM EDTA, fixed in PBS/2% paraformaldehyde, and stained with PE-conjugated anti-mouse I-A/I-E monoclonal antibody M5/114.15.2 (Pharmingen/Becton Dickinson) or APC-conjugated anti-mouse CD86 (B7-2) monoclonal antibody GL1 (Southern Biotechnology) for 30 min on ice in PBS containing 2% BSA (FLUKA) for cell surface labelling. Alternatively, cells were permeabilized and stained in PBS/2% BSA/0,1% saponin for 30 min on ice for total cell surface and intracellular labelling. Control cells were either not stained or stained with the corresponding isotype control antibodies as indicated. After washing, cells were measured using a FACSCalibur™ (Becton Dickinson) and analyzed using FCS Express (De Novo Software).

Cell sorting, lysis and Western blotting

Transduced DCs were harvested in PBS containing 2 mM EDTA. EGFP-negative and EGFP-positive cells were sorted with the FACSVantage™ SE, washed two times with PBS and lysed at room temperature in SDS-sample buffer (2% SDS, 62,5 mM Tris/HCl pH 6,8 and 10% glycerol). Lysates were passed five times through a 25G needle and centrifuged for 5 min at 13.000x*g*. Samples were either kept at room temperature for analysis of peptide loaded MHCII or heated for 5 min at 100 °C for analysis of total MHCII-β. Proteins were separated by SDS-PAGE, transferred to PVDF paper and immuno-blotted. MHCII was labelled with rabbit polyclonal antibody directed against the cytoplasmic domain of MHC-β (kindly provided by Dr N. Barois [35]) followed by horseradish peroxidase (HRP) conjugated goat-anti rabbit antibodies (Pierce). HRP activity was detected on film using Supersignal West Pico Chemiluminescent Substrate (Pierce).

Acknowledgements

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Endosomally stored MHC class II does not contribute to antigen presentation by dendritic cells at inflammatory conditions

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Abstract

Major histocompatibility complex (MHC) class II (MHCII) is constitutively expressed by immature dendritic cells (DC), but has a short half-life as a consequence of its transport to and degradation in lysosomes. For its transfer to lysosomes, MHCII is actively sorted to the intraluminal vesicles (ILV) of multivesicular bodies (MVB), a process driven by its ubiquitination. ILV have, besides their role as intermediate compartment in lysosomal transfer, also been proposed to function as a site for MHCII antigen loading and temporal storage. In that scenario, DC would recruit antigen-loaded MHCII to the cell surface in response to a maturation stimulus by allowing ILV to fuse back with the MVB delimiting membrane. Other studies however, explained the increase in cell surface expression during DC maturation by transient upregulation of MHCII synthesis and reduced sorting of newly synthesized MHCII to lysosomes. Here, we have characterized the relative contributions from the biosynthetic and endocytic pathways and found that the vast majority of antigen loaded MHCII that is stably expressed at the plasma membrane by mature DC is synthesized after exposure to inflammatory stimuli. Pre-existing endosomal MHCII contributed only when it was not yet sorted to ILV at the moment of DC activation. Together with previous records, our current data are consistent with a model in which passage of MHCII through ILV is not required for antigen loading in maturing DC, and in which sorting to ILV in immature DC provides a one way ticket for lysosomal degradation.

Introduction

Dendritic cells (DC) are professional antigen presenting cells with a unique capacity to process and derive peptides from exogenously acquired antigens for presentation by MHC class II (MHCII) to activate naïve T cells. DC display peptide loaded MHCII (pMHCII) complexes in an efficient manner only when activated by danger signals, e.g. through Toll-like receptors. Activated DC also increase their

expression of costimulatory receptors and cytokines, and migrate to draining lymph nodes where they may encounter T cells that recognize specific pMHCII complexes [1, 2]. How MHCII peptide loading and cell surface expression of pMHCII complexes are regulated during DC activation is not completely resolved and a matter of debate (reviewed in 10).

MHCII is synthesized at the endoplasmatic reticulum where it associates with invariant chain (Ii) [3]. This chaperone prevents premature peptide loading of MHCII by occupying its peptide binding groove [4] and contains sorting information at its cytoplasmic domain which guides the complex via the Golgi and plasma membrane to the endocytic pathway [5]. After arriving at endosomes, Ii is degraded in a stepwise fashion, eventually resulting in removal of the Ii contained sorting signals [6, 7]. An Ii derived peptide (CLIP) fragment remains in the MHCII peptide binding groove until substituted by other peptides that are present within the endosomal milieu, in a process that is facilitated by the accessory molecule DM [8]. We recently demonstrated that Ii degradation and peptide loading of MHCII are constitutive processes that occur equally efficient in immature DC (iDC) and mature DC (mDC) [9]. We and others also showed that MHCII is ubiquitinated in iDC and that this modification drives active sorting of MHCII to the intraluminal vesicles (ILV) of multivesicular bodies (MVB) [9-16], probably through interactions with the ESCRT (endosomal sorting complex required for transport) [10]. A portion of pMHCII fails to sort to ILV directly after its generation at the endosomal/lysosomal compartment and reaches the plasma membrane of iDC. These molecules may be important for antigen presentation by tolerogenic iDC [17]. In iDC however, surface MHCII has a short half-life due to endocytosis, which can be driven either by a MHCII-β chain encoded di-leucine motif [18] or the above mentioned ubiquitin tag [12, 13]. Endocytosed MHCII then encounters a second opportunity for ubiquitination driven sorting at MVB [12, 13]. Once sorted to ILV, MHCII is targeted for degradation when MVB fuse with lysosomes. In this way, iDC continuously generate MHCII molecules loaded with self-peptides that are not stably expressed at the plasma membrane. In contrast, DC that are activated by inflammatory stimuli establish stable surface expression of MHCII by abrogating ubiquitination of MHCII [11-13, 19, 20]. At these conditions MHCII is also loaded with peptides from phagocytosed/endocytosed pathogens. Stable cell surface expression of MHCII is achieved, at least in part,

through down-regulation of the MHCII ubiquitinating enzyme MARCH-I [19, 21]. In addition, CD83 has recently been demonstrated to enhance MHCII expression by preventing association of MHCII with MARCH-I [22].

Major questions remain regarding the origin and trajectory of MHCII that is stably expressed at the plasma membrane of maturing DC. Upon activation, conventional DC transiently increase and then shut off MHCII synthesis along their maturation program [23-28]. Drugs that interfere with the biosynthetic pathway, such as brefeldin A or cycloheximide, prevented the increase of MHCII surface expression by maturing DC, fitting the idea that the majority of cell surface expressed MHCII of mDC is synthesized during DC maturation [23]. On the other hand, electron microscopy studies showed that for iDC at steady state ~65% of MHCII located at MVB, of which the majority (~80%) was associated with ILV [29]. Early after activation, DC form endosomal tubules that carry MHCII from endosomes to the plasma membrane [29-33]. ILV disappear concomitant with the appearance of these tubules [29]. This suggested that MHCII molecules in iDC may be temporarily stored at ILV and recruited at inflammatory conditions by back-fusion of ILV with the MVB delimiting membrane, thereby supplying MHCII from this source for cell surface expression [34, 35]. In another study MHCII was found to interact with DM at ILV rather than at the MVB delimiting membrane [36], suggesting that peptide loading may preferentially occur at ILV and supporting the idea that pMHCII may predominantly be recruited from ILV. This model is however inconsistent with our previous observations in iDC, that ubiquitination of MHCII requires prior degradation of its associated Ii and that ubiquitination is preceded by peptide loading. The latter observation suggests that MHCII is already peptide loaded prior to its ubiquitin driven sorting to ILV [13]. At inflammatory conditions it does not seem beneficial, and perhaps even dangerous, to recruit MHCII from ILV that was already stably loaded with self-peptide and sorted to those ILV prior to DC activation.

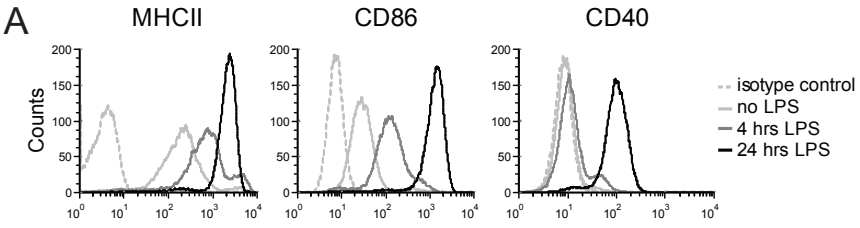
The contradictions between the two models prompted us to determine the relative contributions of newly synthesized versus endosomally stored MHCII for antigen presentation by maturing DC. We found that maturing DC mainly use newly synthesized MHCII and not endosomally stored MHCII to present antigen. A significant contribution from an endosomal source could be observed only when MHCII was accumulated by artificial means at the endosomal delimiting

membrane before application of an inflammatory stimulus. These data indicate that at physiological conditions, the majority of MHCII molecules at ILV are not recruited to the cell surface for antigen presentation but are rather destined for lysosomal degradation.

Results

Surface expression of MHCII in maturing DC changes with synthesis and turnover rates

Cultured DC dramatically increased their surface expression of MHCII, CD86 and CD40 during LPS induced maturation (Figure 1A). Analysis of the subcellular distribution of MHCII in iDC by immuno-electron microscopy (Figure S1) revealed that ~58% of total MHCII is located at MVB and only ~7% at the plasma membrane (Figure 1B). An inverse distribution pattern was observed for LPS activated mDC. In addition to this redistribution, the absolute amount of total cellular MHCII was increased in response to LPS (Figure 1C). These data are consistent with previous reports [29] and may be explained by alternate sorting to lysosomes [12, 13] and/or to a temporal increase in the biosynthesis of MHCII [23-28]. In these and other studies biosynthesis was often measured by metabolic labeling with ^{35}S-amino acids. We and others found that metabolic labelling of DC causes a broad range of non-specific side-effects, including DC activation. This is a direct consequence of contaminating bacterial products present within the ^{35}S-met/cys label (that is generated in bacteria grown on ^{35}S, contaminating this reagents with a plethora of PAMPS), as well as of the required temporal serum starvation. To circumvent such undesired side effects, we analyzed MHCII synthesis by applying Endoglycosidase H (EndoH) after cell lysis. EndoH removes high mannose glycans from newly synthesized glycoproteins, but not from complex glycosylated proteins which have traversed the Golgi and can therefore be used to discriminate newly synthesized MHCII. The amount of EndoH-sensitive MHCII increased during the first 2 hrs of LPS treatment, and slowly decreased thereafter with only minimal contributions after 16 hrs (Figure 1C). These data confirm that maturing DC transiently upregulate their synthesis of MHCII.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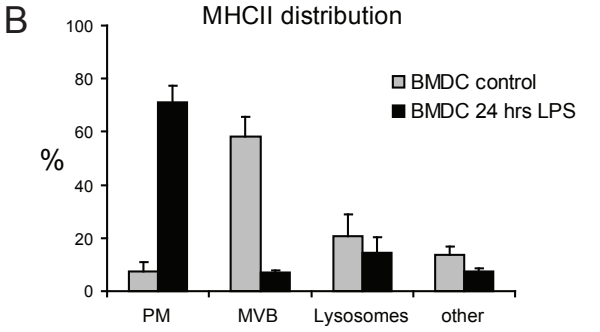
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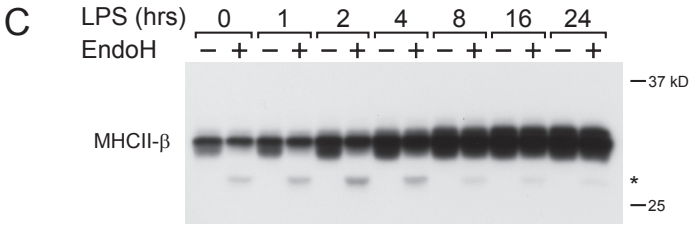
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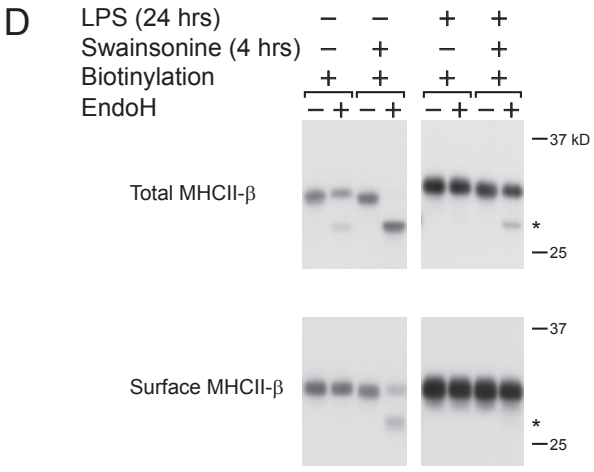
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To determine the relative contribution of newly synthesized MHCII to the total of cell surface expressed MHCII, DC were cultured with swainsonine, a selective mannosidase-II inhibitor that neither interferes with post-endoplasmic reticulum (ER) transport of membrane proteins nor with MHCII restricted antigen presentation [37, 38]. If any process relevant to MHCII traffic would have been affected by swainsonine this should have been reflected by changes in the cell surface expression of these molecules. However, cell surface expression of MHCII, the costimulatory molecule CD86, and the activation marker CD40, were not affected by the presence of swainsonine, neither in immature DC nor in LPS activated DC (Figure S2). This indicates that swainsonine could safely be used in our studies, irrespective of the itinerary of MHCII during DC maturation. In the presence of swainsonine a cohort of MHCII was synthesized that remained EndoH sensitive after passage through the Golgi. Plasma membrane associated MHCII was then selectively marked by cell surface biotinylation and selectively extracted from lysed cells using neutravidin conjugated beads (Figure 1D). As expected, only EndoH resistant MHCII reached the cell surface in the absence of swainsonine. Already after 4 hrs with swainsonine, ~50% of MHCII that was surface exposed by iDC became sensitive to EndoH, reflecting a relative rapid turnover. In contrast, fully matured DC synthesized only little MHCII during a 4 hrs incubation with swainsonine. This population did not contribute significantly to cell surface exposed MHCII, consistent with a slow turnover in mDC.

Together these data are in agreement with the concept that both a

Figure 1 (left). Changes in surface expression, subcellular distribution and synthesis of MHCII during DC activation

(A) Flow cytometric analysis of surface expression of MHCII and the co-stimulatory markers CD86 and CD40 on DC after 0, 4 and 24 hrs of LPS treatment. **(B)** Ultrathin cryosections of immature and 24 hrs LPS matured DC were immuno-gold-labeled for MHCII and analyzed by electron microscopy as exemplified in supplementary figure 1. Plotted is the relative distributions of gold particles over plasma membrane (PM), MVB, lysosomes and collective other locations (ER, Golgi complex, cytoplasm, mitochondria and the nucleus) (n=3 ± SD). **(C)** DC were incubated with LPS for the indicated time. Cell lysates were treated with or without EndoH and immunoblotted for MHCII-β chain (MHCII-β). EndoH sensitive MHCII-β which runs just above the 25 kD marker, is indicative for the relative amount of newly synthesized ER-associated MHCII. **(D)** Immature and 24 hrs LPS treated DC were cultured for 4 hrs with or without swainsonine as indicated. Intact cells were then surface biotinylated at 0 °C and lysed. Total and isolated biotin-labeled MHCII were treated with or without EndoH and imunnoblotted for MHCII-β. Asterisks indicate unglycosylated MHCII-β.

transient increase in synthesis and a prolonged half-life of MHCII elevate the expression of MHCII during DC maturation.

Plasma membrane-deposited MHCII in maturing DC is newly synthesized

To determine the major source of MHCII that is stably expressed at the cell surface upon activation, DC were treated with LPS in the presence or absence of swainsonine. Cell surface biotinylated MHCII was then isolated, EndoH treated and immuno-blotted for MHCII-β chain (Figure 2A). After 4 hrs with both LPS and swainsonine ~57±7% (n=3) of MHCII at the plasma membrane was EndoH sensitive, and ~76±6% (n=4) after 8 hrs (Figure 2A, lanes 11-14). When swainsonine was present only during the last 4 hrs of an 8 hrs LPS stimulation, ~45% of surface MHCII was EndoH sensitive (Figure 2A, lanes 15-16), demonstrating that the time frames 0-4 hrs and 4-8 hrs within the 8 hrs of activation contributed about equally to newly synthesized surface MHCII. The relatively much higher turnover of MHCII in iDC was confirmed by the near absence of EndoH resistant MHCII after 8 hrs culture with swainsonine only (Figure 2A, lanes 5-6).

These data already indicate a crucial role for MHCII synthesis in building the surface exposed pool of MHCII in maturing cells. To validate this further, DC were treated with LPS and/or swainsonine for 4 hrs, while ER-exit was inhibited with brefeldin A (BFA) (Figure 2B). Indeed, appearance at the plasma membrane of newly synthesized MHCII, which should remain EndoH sensitive in the presence of swainsonine, was completely abrogated by BFA, both in the presence and absence of LPS. Cell surface expression of MHCII increased >2 fold already after 4 hrs of LPS as determined by surface biotinylation (Figure 2B) and flow cytometry (Figure 2C). This increase was almost completely abrogated by BFA, again suggesting that the increase in surface MHCII as observed for maturing DC is dependent on *de novo* synthesis.

Recruitment to the cell surface of MHCII from the endosomal delimiting membrane

Our electron microscopy data show that in iDC ~58% of total MHCII resides at MVB (Figure 1B). Of this large MHCII population, as much as ~85% was at ILV and the remainder at delimiting membranes (Figure 3B,C). We [29] and others [36]

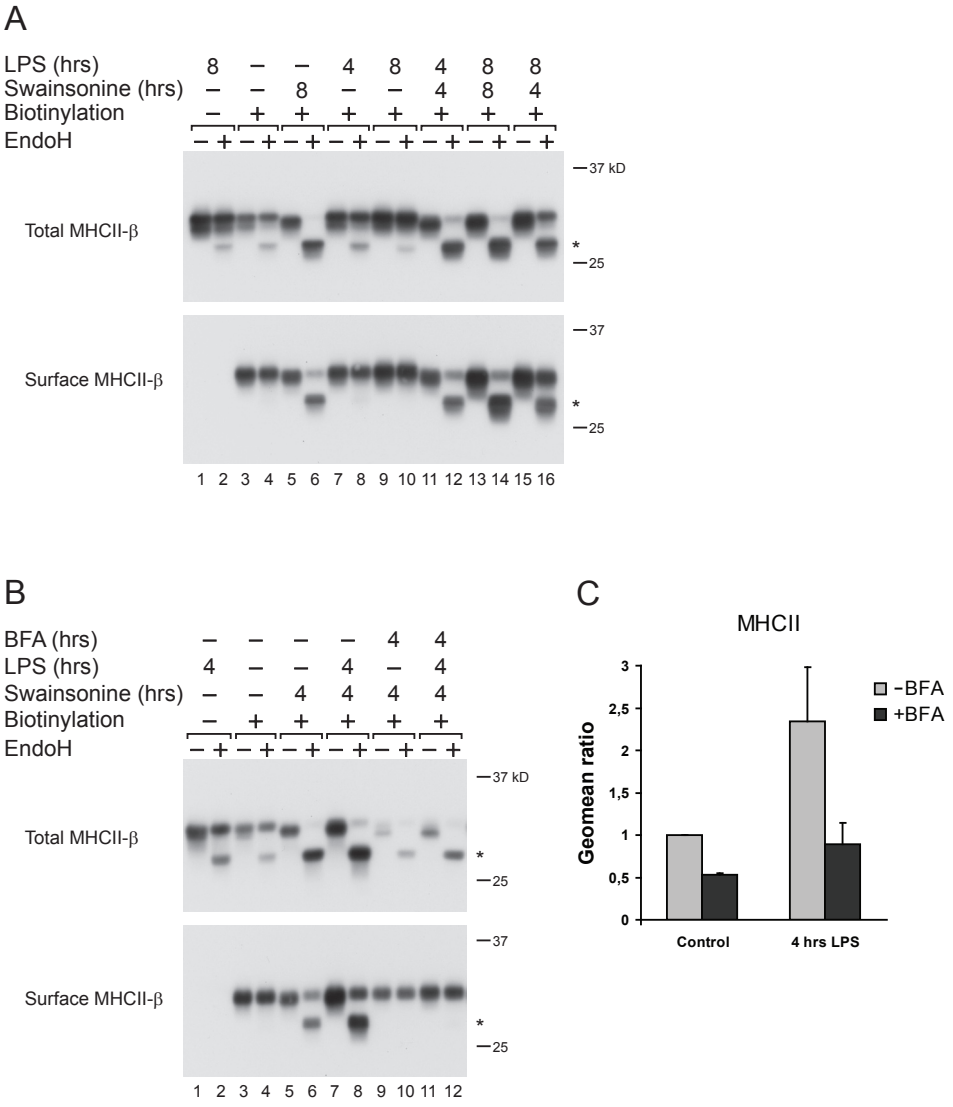


Figure 2. Surface MHCII on mature DC is synthesized after DC activation
(A) DC were activated with LPS for 4 or 8 hrs in the presence or absence of swainsonine as indicated. Intact cells were then surface biotinylated at 0 °C and lysed. Total and isolated biotin-labeled MHCII were treated with or without EndoH and immunoblotted for MHCII-β. No MHCII-β was extracted when cells were not biotinylated (lanes 1 and 2). **(B)** Cells were treated and analyzed as in (A) except that BFA was present when indicated. Asterisks indicate unglycosylated MHCII-β. **(C)** Cells were treated with LPS and/or BFA as indicated and surface expression of MHCII was analyzed with flow cytometry. The geomean of untreated cells was set to 1, and data are expressed relative to this value (± SD). Data from three independent experiments were pooled.

have proposed previously that during DC maturation MHCII may be recruited from ILV to the plasma membrane. Recruitment from ILV would require fusion of ILV back with the endosomal delimiting membrane. Alternatively, recruitment of MHCII from the endosomal delimiting membrane directly after processing of associated Ii and peptide loading at that location would not require ILV back-fusion.

To investigate the potential contribution of MHCII from these two sub-endosomal compartments we made use of an artificial condition at which a cohort of newly synthesized MHCII could be transiently trapped at the MVB delimiting membrane prior to DC activation. Hereto, we relied on our previous demonstration that when iDC are cultured in glutamine-containing media, endosome acidification and consequently also pH-dependent degradation of Ii are inhibited [9]. In fact, in many laboratories DC are cultured in glutamine-containing medium, yielding contradictory results. When cells are cultured in 4 mM glutamine, 2 mM ammonia is formed within 2-4 days, resulting in interference with Ii processing [9]. In our current study we used exactly that concentration of ammonia (2 mM) as a defined control condition to artificially increase the amount of MHCII-Ii complexes at the MVB delimiting membrane. This experimental condition was thus used to accumulate a normally much more transient pool of MHCII at the endosomal delimiting membranes. Of note, at a concentration of 2 mM, ammonia had only a minor effect on the endosomal/lysosomal pH (from pH 5.0 to 5.6), which was instantaneously recovered upon ammonia removal [9]. Moreover, the ammonia concentration used here is much lower than in numerous other studies (10-50 mM) where ammonia was used to fully neutralize the endosomal/lysosomal pH and interfere with Ii processing or MHCII presentation.

As ubiquitination of MHCII and thus its sorting to ILV require prior degradation of Ii [10, 13], we anticipated that we could artificially accumulate MHCII-Ii complexes at the endosomal delimiting membrane with 2 mM NH_4Cl (Figure 3A). Indeed, electron microscopic analysis of the distribution of MHCII within MVB revealed that of all the MHCII at MVB as much as ~74% resided at the delimiting membrane after 18 hrs NH_4Cl treatment, while only ~15% was at the delimiting membrane in the absence of NH_4Cl (Figure 3B,C). With NH_4Cl, both MHCII and intermediate Ii degradation products accumulated, while

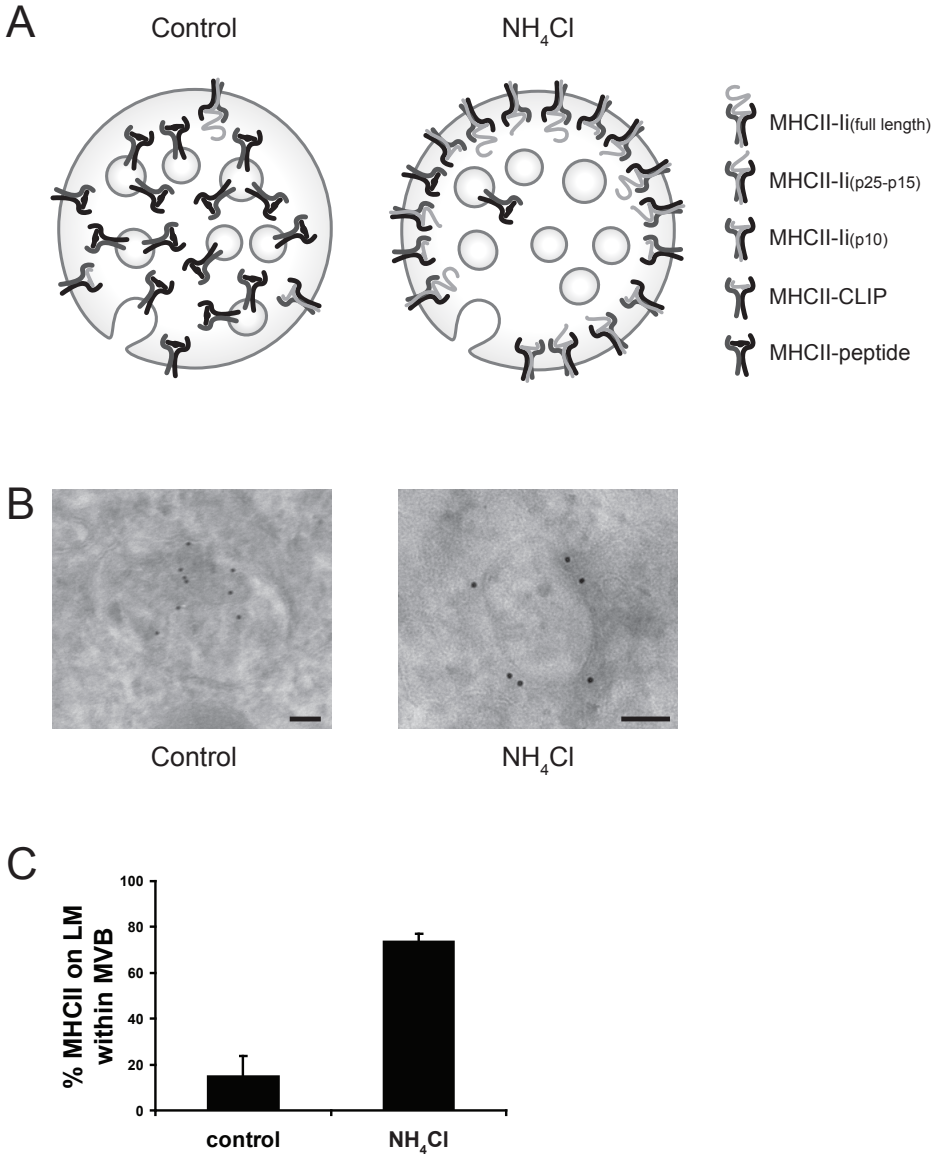


Figure 3. Redistribution of MHCII within MVB by NH_4Cl
DC were incubated for 18 hrs in the presence or absence of 2 mM NH_4Cl. **(A)** NH_4Cl slightly raised the pH of endosomal compartments [9], therewith preventing proteolysis of MHCII associated Ii and hence also downstream ubiquitination and sorting to ILV of MHCII. **(B)** Examples of MVB in iDC that were cultured at control conditions or for 18 hrs in the presence of NH_4Cl, and the distributions of MHCII therein as determined by immuno-electron microscopy. Bars, 100 nm. **(C)** The distribution of MHCII at MVB as exemplified in (B); delimiting membrane (LM) versus the total (ILV + delimiting membrane) in control and NH_4Cl treated cells.

ubiquitination of MHCII was reduced (Figure 4A,C) [9]. Accumulation of the full-length Ii splice variants p41 and p31 was less apparent, probably due to their relative abundance in the ER [39].

Anticipating that interference with Ii processing by NH_4Cl would be reversible, we used this as a tool to study whether MHCII would be recruited from the endosomal delimiting membrane to the plasma membrane in response to DC activation, and to compare the characteristics of this pool with that of newly synthesized MHCII. When NH_4Cl treated cells were washed and chased in the absence of NH_4Cl, the previously accumulated intermediate degradation products of Ii disappeared, reaching control levels at ~8 hrs (Figure 4A). Concomitant with Ii degradation we observed a transient increase in MHCII peptide loading, as determined by the appearance of SDS-resistant pMHCII complexes. This method thus allowed us to synchronize Ii degradation and peptide-loading of newly synthesized MHCII. After 18 hrs culture with NH_4Cl, cell surface expression of MHCII by iDC was reduced twofold fold (Figure 4B), despite the increase of total cellular MHCII (Figure 4A). This is probably due to prolonged association of MHCII with full length Ii or intermediate Ii degradation products, which contain two di-leucine motifs that drive fast clathrin-mediated endocytosis [5, 40, 41]. Upon removal of NH_4Cl, surface expression of MHCII increased almost 6 fold after 3-6 hrs of chase and 3 fold as compared to control cells that had not been exposed to NH_4Cl. This is a substantial increase given that similar surface expression levels were achieved for DC that were activated with LPS for 4 hrs. NH_4Cl treated DC were however still immature as established by their low CD40 expression. The surface appearance of MHCII after NH_4Cl removal correlated with the degradation of accumulated Ii (intermediates) and with the appearance of SDS-stable pMHCII complexes. This is consistent with the notion that surface expression of MHCII in iDC is prolonged by its release from endocytosis motif containing Ii. Surface MHCII started to decline again after 8 hrs of NH_4Cl removal (Figure 4B), probably as a consequence of sorting to lysosomes. Previously we demonstrated that only pMHCII is ubiquitinated and that degradation of associated Ii is necessary before ubiquitination of MHCII can occur [13]. Consistent with this we found that ubiquitination of MHCII was restored upon NH_4Cl removal and even increased above control levels (Figure 4C). Again this correlates with the kinetics of disappearance of MHCII from the cell surface

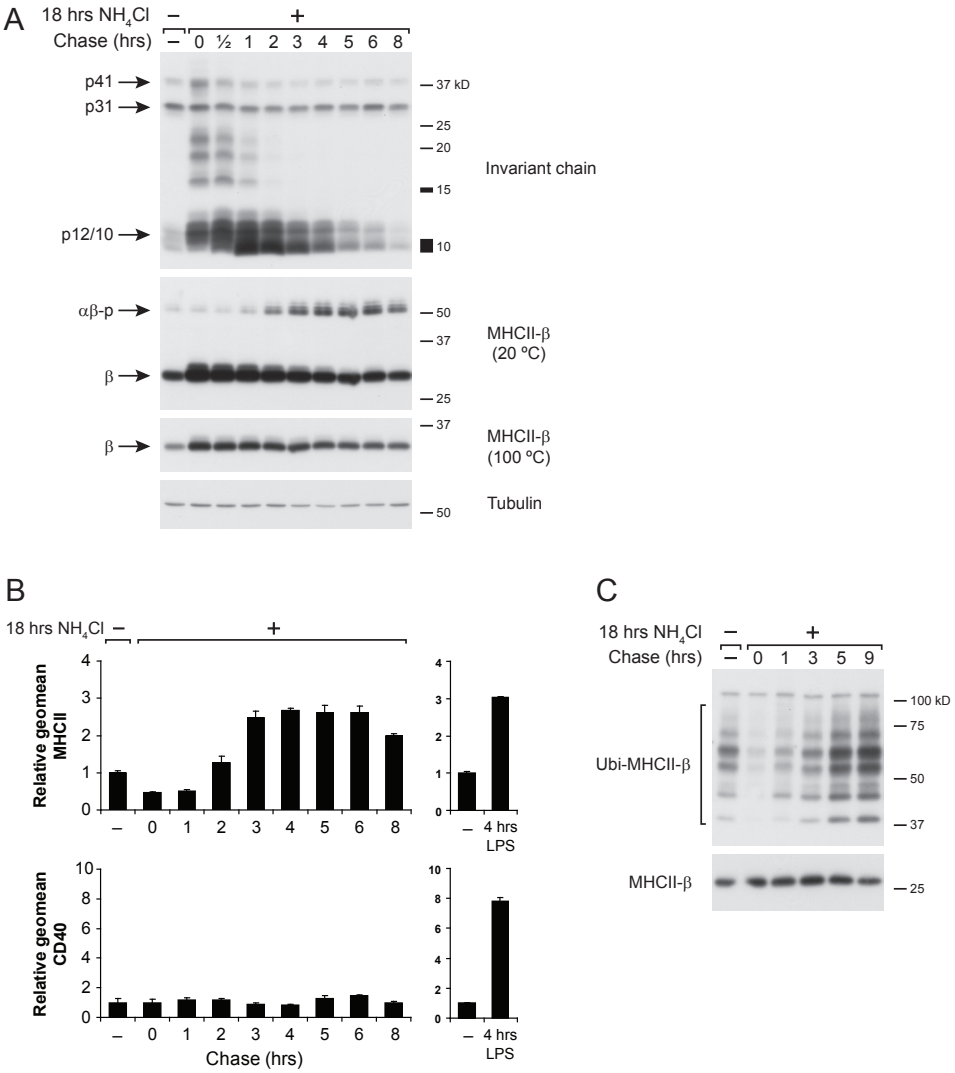


Figure 4. Processing of MHCII-Ii during recovery from a NH_4Cl treatment
(A) MHCII-Ii complexes were accumulated in iDC during 18 hrs in 2 mM NH_4Cl and chased up to 8 hrs after NH_4Cl removal as indicated. Ii, MHCII-β and tubulin (loading control) were analyzed by immuno-blotting as indicated. p41 and p31 indicate full length Ii splice variants; p12/10 and bands between 15-25 kD indicate Ii intermediate degradation products. Total MHCII-β is reflected by 100 °C sample and SDS stable pMHCII (αβ-p) by 20 °C sample. **(B)** iDC were treated as in (A) after which cell surface expression of MHCII and CD40 (DC activation marker) was determined by flow cytometry. The geomean of untreated cells was set to 1, and data are expressed relative to this value (± SD) **(C)** DC were treated as in (A), after which MHCII was immuno-precipitated from cell lysates and analyzed for total MHCII-β and ubiquitin conjugated MHCII-β by immuno-blotting. Figures are representative for at least three independent experiments.

(Figure 4B). We conclude that this procedure enabled us to release a cohort of newly synthesized pMHCII that had been accumulated at the endosomal delimiting membrane.

Figure 2 showed that the increase in cell surface expression of MHCII by maturing DC can be prevented with BFA, and we interpreted this to be due to interference with the exit of newly synthesized MHCII from the ER. However, BFA also has dramatic effects on endosome morphology and minor kinetic effects on the recycling of some receptors to the plasma membrane [42-44]. To test the possibility that BFA may interfere with the transfer of MHCII from endosomes to the plasma membrane, experiments were performed wherein MHCII was first accumulated at the MVB delimiting membrane using NH_4Cl. Endosomal MHCII was then released from this block in the absence or presence of LPS and/or BFA (Figure 5). In the absence of a previous NH_4Cl block, the amount of pMHCII complexes, as determined by SDS stability at 20 °C, dramatically increased during 24 hrs in the presence of LPS. This was not observed when BFA was present, consistent with the previous observation that antigen presentation by maturing DC primarily depends on newly synthesized MHCII (Figure 2) [27]. The inhibitory effect of BFA was reversible as demonstrated in experiments where BFA was removed after 4 hrs and the cells chased for another 18 hrs (lanes marked 4+ in Figure 5A). Interestingly, when MHCII-Ii was first accumulated at the delimiting membrane of endosomes in iDC by the presence of NH_4Cl, the amount of pMHCII increased during a subsequent chase without NH_4Cl in the presence of BFA and LPS. This demonstrates that upon DC activation a substantial amount of pMHCII could be generated from the accumulated MHCII without a contribution from molecules synthesized after the application of the maturation stimulus. We also conclude that BFA did not interfere with peptide loading. At these conditions also cell surface expression of MHCII was determined by flow cytometry (Figure 5B). BFA efficiently prevented maturing DC to increase their MHCII surface expression at control conditions. However, when MHCII-Ii was first accumulated at endosomes by NH_4Cl prior to DC activation, surface exposed MHCII increased irrespective of the presence of BFA. Similar observations were made for MHCII that was loaded with an exogenously added peptide ($E\alpha_{52-68}$) and detected with a MHCII-$E\alpha_{52-68}$ complex specific monoclonal antibody (Y-Ae) [45]. Again, these data illustrate that BFA did not interfere with MHCII peptide loading and recruitment to the cell surface.

Collectively, our data demonstrate that MHCII on the surface of mDC is normally synthesized during DC maturation rather than derived from a pre-existing storage pool formed in MVB at the DC immature stage. The data also suggest, together with the previous acquired knowledge [12, 13] that MHCII is ubiquitinated after peptide loading and that ubiquitin mediated sorting into ILV

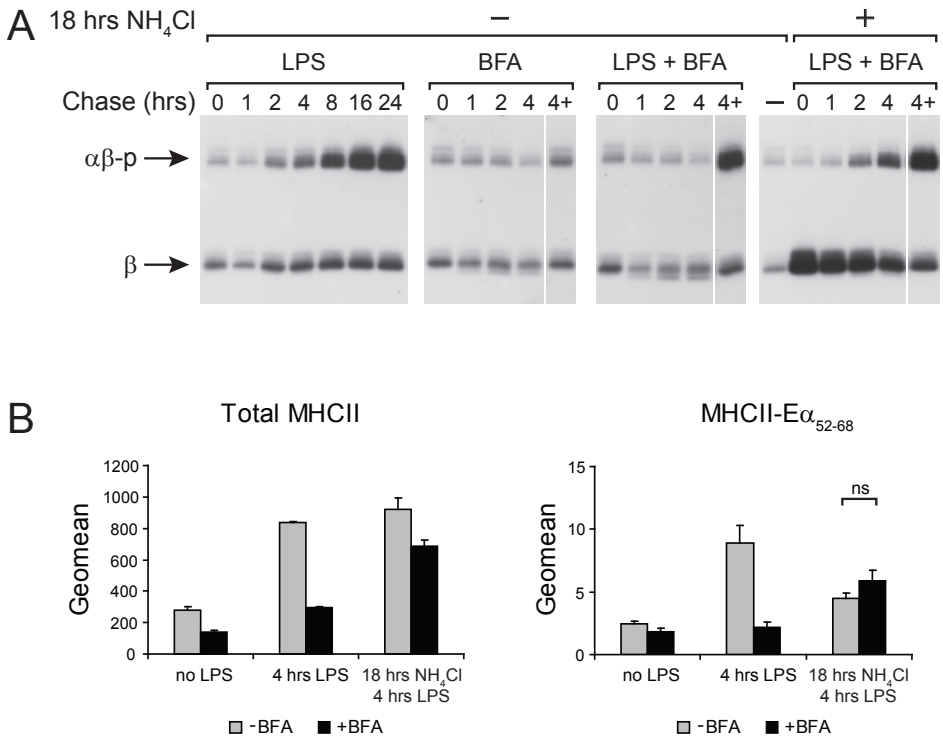


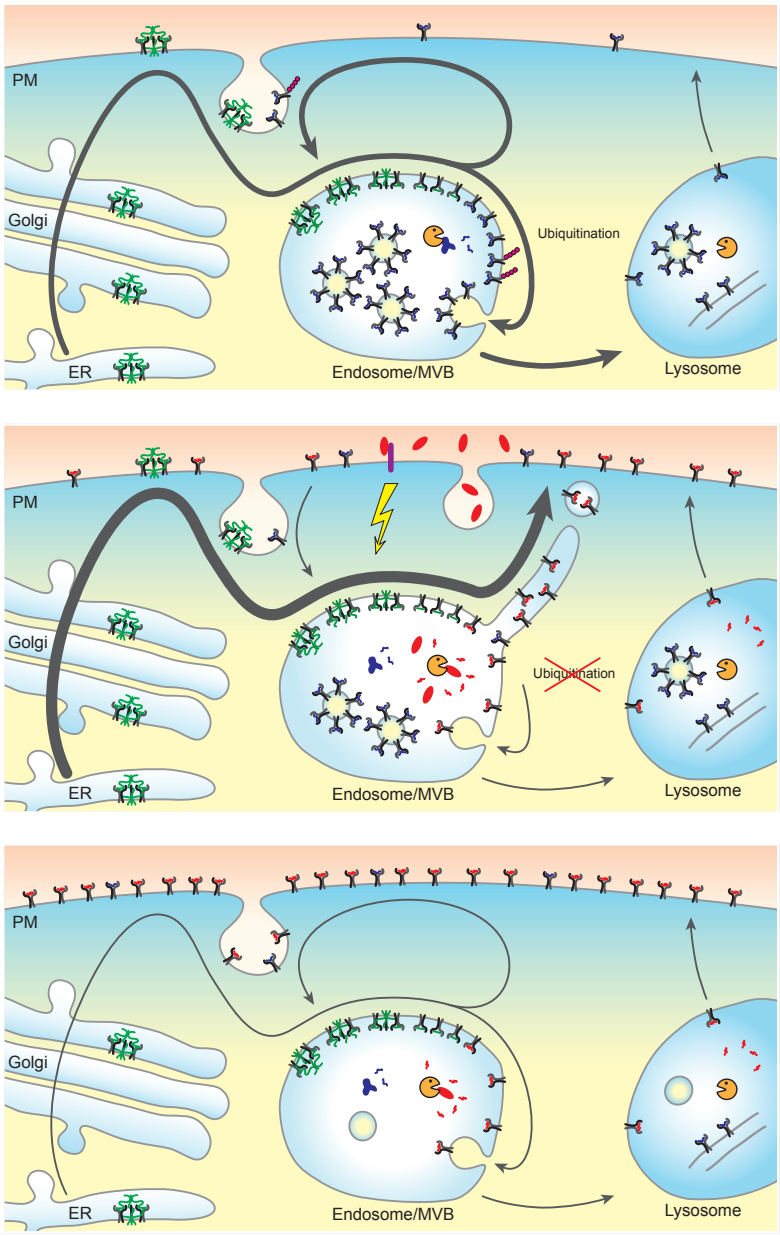
Figure 5. Peptide loading and transport to the cell surface of endosomal MHCII are not affected by BFA

(A) DC were treated with NH_4Cl, LPS and BFA according to the indicated schedule, lysed in SDS containing buffer at 20 ºC to sustain pMHCII complexes (αβ-p) and immuno-blotted for MHCII-β. Samples marked with "4+" represent positive controls of DC that were incubated with BFA ± LPS for 4 hrs and chased for 18 hrs in the absence of BFA. **(B)** DC were cultured with NH_4Cl as indicated and loaded with $E\alpha_{52\text{-}68}$ peptide for 1.5 hrs. DC were then chased for 4 hrs in the absence of NH_4Cl and presence of LPS and/or BFA as indicated. Cell surface expression of total MHCII or $E\alpha_{52\text{-}68}$ loaded MHCII were analyzed by flow cytometry and indicated as the geomean (± SD). Data are representative of three independent experiments. LPS induced surface expression of MHCII and $E\alpha_{52\text{-}68}$ loaded MHCII was blocked by BFA at control conditions, indicating the requirement of newly synthesized MHCII, but not when MHCII-Ii was first accumulated in endosomes in the presence of NH_4Cl ($p=0.08$). All figures are representative for at least three independent experiments.

Immature DC

maturing DC

mature DC



MHCII-Ii
MHCII-CLIP
MHCII-self peptide
ubiquitinated MHCII
MHCII-pathogen peptide
proteases
pathogen
pathogen recognition receptor
self protein
signal transduction

is abrogated in maturing DC, that passage of MHCII through ILV is not required for peptide loading. In conclusion (Figure 6), DC activation results in a temporal increase in MHCII synthesis. This newly synthesized pool is then transferred to the endosomal/lysosomal delimiting membranes where it is loaded with peptides, and subsequently transferred from these delimiting membranes via membrane tubules/vesicles that derive thereof to the plasma membrane. Pre-existing MHCII at ILV that was synthesized during the DC's immature stage is transferred to lysosomes for degradation and not used for antigen presentation during DC maturation.

Figure 6 (left). Model for MHCII traffic at different stages of DC maturation
MHCII-Ii complexes are synthesized at the endoplasmatic reticulum and transferred via the Golgi and plasma membrane to the endocytic pathway with the aid of Ii encoded sorting signals [5]. After arrival at endosomes, Ii is degraded in a stepwise fashion, eventually resulting in removal of the Ii contained sorting signals [6, 7]. CLIP is then substituted from MHCII by other peptides that are present within the endosomal lumen in a constitutive manner that occurs equally efficient in iDC and activated DC [9]. Only in iDC, however, pMHCII is ubiquitinated driving active sorting to ILV of MVB [9-16], probably through interactions of ubiquitinated MHCII with ESCRT [10]. The relevant ubiquitin ligase MARCH-I is mainly localized at endosomes [53, 54], suggesting that ubiquitination occurs directly after peptide loading in the same compartment. A portion of the pMHCII, however, escapes sorting and reaches the plasma membrane of iDC. In this event ubiquitination also enhances its endocytosis for a second opportunity of sorting at MVB [12, 13]. MHCII that is sorted to ILV cannot escape via recycling endosomes, and as a consequence is targeted for degradation in lysosomes. In this way, MHCII molecules in iDC, which are normally loaded with self-peptides (in blue), are inefficiently expressed at the plasma membrane, where they may function to maintain immune tolerance [17]. In maturing DC (middle panel), synthesis of MHCII is temporarily upregulated (as indicated by the thickness of the arrow). At the same time, ubiquitination of newly synthesized MHCII is abrogated, after which its active sorting to ILV and lysosomal degradation is disabled [11-13, 19, 20]. Instead, these newly synthesized MHCII molecules, now potentially loaded with peptides from endocytosed/phagocytosed pathogens (in red), are transferred from the endosomal delimiting membrane, via tubular intermediates and transport vesicles to the plasma membrane [29-31]. Although these tubules and transport vesicles are functional intermediates for the transfer of newly synthesized MHCII that travels via the limiting membrane of endocytic compartments to the plasma membrane, they do not seem to selectively recruit MHCII [29]. Some MHCII may still be incorporated into ILV in the absence of ubiquitination [12, 13], but this does not seem to be selective since the labelling density (number of MHCII molecules per µm membrane) at ILV is comparable to that at the MVB delimiting membrane in mature DC [29]. Some MHCII may be transferred from the MVB delimiting membrane to the lysosomal delimiting membrane, and from there to the plasma membrane for presentation of lysosomally generated peptides. MHCII that was already present at ILV prior to the DC maturation stimulus is transferred to lysosomes for degradation. Fully matured DC (bottom panel) shut down their synthesis of MHCII, leaving the cell with a plasma membrane pool of MHCII that had predominantly been synthesized and loaded with peptide during pathogen contact.

Discussion

The origin of MHCII that is used by DC for antigen presentation at inflammatory conditions had remained unclear. One model suggested that MHCII responsible for antigen presentation in mDC would be recruited from ILV [29], whereas another model prefers a major contribution from newly synthesized MHCII [23]. We here provide evidence that DC use newly synthesized rather than their endosomally stored MHCII for antigen presentation at inflammatory conditions (Figure 6). In activated DC newly synthesized MHCII first has to arrive at the delimiting membranes of the endosomal compartment for Ii processing and peptide loading, before it can be stably expressed at the plasma membrane. Also MHCII that is already synthesized prior to DC activation can be stably recruited to the cell surface, but only when not yet sorted to ILV at the time of DC activation.

We confirmed previously reported changes in MHCII synthesis and turnover during DC maturation [23-28] by demonstrating transient upregulation of MHCII synthesis during the first hours of DC maturation, followed by residual synthesis in fully matured DC (Figure 1C,D). Other studies used ^{35}S-methionine/cysteine metabolic labeling experiments to follow MHCII synthesis and processing in DC. Using such assays, however, polluting bacterial substances in commercially available ^{35}S-methionine and serum starvation may trigger DC maturation, thus intervening with MHCII dynamics in immature DC. Our alternative approach that uses swainsonine to prevent complex glycosylation, in combination with an EndoH assay enabled us to accurately identify newly synthesized MHCII at the cell surface. Strikingly, we found that ~50% of MHCII at the cell surface was newly synthesized already after 4 hrs maturation, and that this biosynthetic contribution was sensitive to the Golgi-disrupting drug BFA, further confirming its biosynthetic nature.

Transfer of endosomally processed MHCII to the plasma membrane was insensitive to BFA as demonstrated in control experiments where MHCII-Ii complexes were first artificially accumulated at the endosomal delimiting membrane by the presence of 2 mM NH_4Cl prior to DC activation. Elsewhere we demonstrated that Ii is degraded equally efficient by immature and mature DC and that Ii processing is a prerequisite for MHCII peptide loading and

ubiquitination, sorting to ILV and lysosomal degradation [9, 13]. We also showed that Ii degradation was restricted in immature DC only when they were cultured in glutamine containing media, yet restored in response to LPS. This artificial behavior could be prevented by culturing DC in media containing a stable di-peptide as a glutamine source, attributing these effects to the glutamine decomposition product NH_4^+. Glutamine containing media have inhibitory effects on Ii processing similar to media supplemented with 2 mM NH_4Cl, the same condition used in the current study to accumulate MHCII-Ii at the endosomal delimiting membrane [9]. Of note, in many studies from other laboratories DC were cultured in glutamine containing media. Such conditions may have contributed to alternative conclusions regarding Ii processing and the fate of MHCII at ILV in iDC versus mDC.

It has been proposed that peptide loading onto MHCII may occur at ILV. This idea was supported by a fluorescence resonance energy transfer assay by which MHCII and DM were shown to interact primarily at ILV rather than at the delimiting membrane of enlarged MVB in transfected HEK293 cells [36]. ILV are completely severed from and unconnected to the limiting membrane [46]. Thus, when peptide would be loaded at ILV, recruitment of pMHCII complexes to the plasma membrane would require ILV to fuse back with the MVB delimiting membrane. It should be noted, however, that although functional roles in back-fusion for lysobisphosphatidic acid, the ESCRT-I subunit Tsg101 and Alix have been proposed [35], actual events of ILV back-fusion have never been demonstrated in live cells. As proposed [29], back-fusion of ILV might even drive the formation of MVB-associated tubules in maturing DC [30-33]. These tubules are functional intermediates for the transfer of newly synthesized MHCII that travels via the delimiting membrane of endocytic compartments. Alternatively, the generation of these tubules in maturing DC can also be explained by the increase in amount of cargo caused by the upregulated synthesis of MHCII and simultaneous interference with its active sorting to ILV.

The hypothesis that MHCII may be peptide loaded at ILV [36] is difficult to reconcile with the mechanism of MHCII sorting at MVB that we published elsewhere [13] or with the data in the current paper. Interference with proteolytic processing of associated Ii prevented ubiquitination of the MHCII-β chain, indicating that ubiquitination depends on prior degradation of associated Ii. In

accordance with this notion, MHCII failed to sort into ILV in cathepsin S deficient intestinal epithelial cells [47] or antigen presenting cells [48]. More directly, we demonstrated that only pMHCII complexes are ubiquitinated and hence actively sorted to ILV [13]. We do not suggest that MARCH-I preferentially recognizes pMHCII over MHCII-CLIP but that, because of the high rate of DM driven CLIP-peptide exchange, ubiquitination effectively occurs only after peptide loading. MHCII in which the ubiquitination site had been mutated was loaded with peptide as efficient as wild type MHCII [12], again suggesting that passage of MHCII through ILV is not required for peptide loading. Of note, incorporation of MHCII to ILV has been reported, although much less efficient, to occur also in the absence of ubiquitination [12, 13]. Furthermore, although the majority of DM is located at the endosomal delimiting membrane, some also resides at ILV [29]. Therefore, the possibility that MHCII peptide loading may also occur at ILV remains open.

MHCII is enriched at ILV only in iDC and consequently contains self peptides. For DCs that are activated by pathogen contact, recruitment of pMHCII that were already generated before pathogen contact can potentially evoke autoimmunity, unless self peptides are exchanged for foreign peptides after the uptake of pathogens. However, given that ubiquitinated MHCII is already associated with high affinity peptides, as determined by their stability in SDS at room temperature [13], it is highly unlikely that pMHCII arriving at ILV can substitute its peptide. On a more general note, based on the above mentioned arguments our data support the idea that incorporation of proteins into ILV is a one way ticket for lysosomal degradation.

Materials and Methods

Cell culture

Bone marrow derived DC were generated from C57BL/6 mice as described [49] with minor modifications. Instead of RPMI-1640 medium supplemented with rmGM-CSF, Iscove's Modified Dulbecco's medium lacking L-glutamine supplemented with 2 mM Ultraglutamine 1 (both from Biowhittaker), 10% heat inactivated FCS (Sigma-Aldrich), 100 IU/ml penicillin and 100 mg/ml

streptomycin (GIBCO), 50 µM β-mercaptoethanol and 30% conditioned medium from GM-CSF producing NIH 3T3 cells (R1) [9, 50] was used. Non-adherent cells were harvested and reseeded at day 7-8 and day 10-13 of culture. Assays were performed at day 10 to 14 of culture. The quality of DC cultures was routinely tested for cell surface expression of CD11c, MHCII, CD86 and CD40 by flow cytometry. In all experiments, cultures contained ≥95% DC as determined by CD11c expression. When indicated, DC were incubated either with 1 µg/ml LPS (Escheria coli, serotype 0111:B4 from Fluka), 2 mM NH_4Cl, 10 µg/ml brefeldin A, 0.3 µg/ml swainsonine (both from Sigma-Aldrich), or 10 µM $E\alpha_{52-68}$ peptide [45] (GenScript).

Cell surface biotinylation

DC were harvested and washed 3 times at 0 °C in phosphate-buffered saline (PBS) and incubated 30 min on ice in 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific). Free biotin was quenched and removed by washing labeled DC once in PBS/100 mM glycine and twice in PBS/50 mM glycine/1% BSA. Cells were then lysed in Triton-X100 containing buffer as described above. Biotinylated proteins were extracted with neutravidin-agarose beads (Pierce) at 4 °C for 1 hr, washed with Triton-X100 buffer and eluted in SDS sample buffer. Isolated biotinylated proteins and total cellular proteins were treated with EndoH and analyzed by immuno-blotting as indicated below.

Immunoprecipitation and blotting

DC were lysed at 0 °C in buffer containing 1% Triton-X100, 1 mM EDTA, 20 mM TRIS/HCl (pH 7.5), 150 mM NaCl, 10 mM N-Ethylmaleimide (Sigma-Aldrich) and complete protease inhibitor mix (Roche). After removal of nuclei by centrifugation, cell lysates were either directly used for immuno-blotting according to standard procedures or processed by immuno-precipitation before immuno-blotting. For immuno-precipitation of MHCII, rat anti-mouse I-A/I-E monoclonal antibody (M5/114.15.2 from BioLegend) was added, incubated for 1 hr at 4 °C and precipitated with Protein G agarose beads (Pierce). Beads were washed and eluted in SDS sample buffer for 5 min at 100 °C. MHCII-β, ubiquitinated MHCII-β, or associated Ii were then separated by SDS-PAGE and detected by immuno-blotting. Rabbit polyclonal antibodies directed against

cytoplasmic domains of mouse MHCII-β and Ii were kindly provided by Dr N. Barois [51] and detected using HRP-conjugated secondary antibodies (Pierce Biotechnology Inc, Rockford IL, USA). HRP-conjugated anti-ubiquitin antibody P4D1 was from Santa Cruz Biotechnology and anti α-tubulin clone DM1A from Sigma-Aldrich. For detection of pMHCII from cell lysates or EndoH treatment, DC were lysed in buffer containing 2% sodium dodecyl sulphate (SDS), 62.5 mM TRIS/HCl (pH 6.8), and 10% glycerol, passed 5 times through a 25G needle and centrifuged 2 min at 13.000x*g*. Samples were kept at room temperature for analysis of pMHCII or heated for 5 min at 100 ºC at reducing conditions for analysis of total MHCII-β. For EndoH treatment, cell lysates were incubated with Endoglycosidase H (New England Biolabs) as recommended by the manufacturer. Immuno-blot signals were detected on film using Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific) and quantified by densitometer scanning.

Flow cytometry

DC were harvested and immunolabeled in suspension for 30 min at 0 ºC in PBS/1% BSA with Allophycocyanin-conjugated anti I-A/I-E (M5/114.15.2), anti-CD11c (N418), Fluorescein isothiocyanate-conjugated anti-CD86/B7-2 (GL1), anti-I-A^b/E$\alpha_{52\text{-}68}$ (Y-Ae) (all from eBiosciences) or Phycoerythrin-conjugated anti-CD40 (3/23 from Southern Biotechnology). Non-specific background staining was determined with corresponding isotype control antibodies. To correct for background of the I-A^b/E$\alpha_{52\text{-}68}$ (Y-Ae) staining, DC not loaded with E$\alpha_{52\text{-}68}$ peptide were used. Labeled cells were analyzed by flow cytometry using a FACSCalibur, CellQuest (Becton Dickenson Biosciences) and FCS Express (De Novo Software).

Immuno-electron microscopy

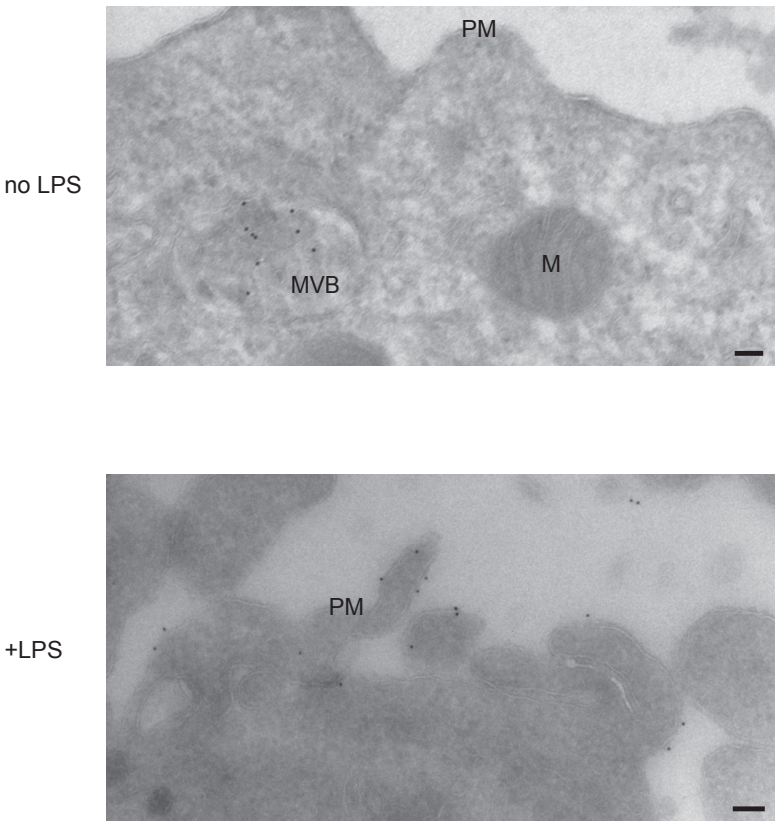
Cells were fixed, and ultrathin cryosections were immuno-gold labelled as described [52]. I-A^b was labeled with M5/114.15.2, followed by rabbit anti-rat IgG (Dako, Denmark) and protein A conjugated to 10 nm gold particles. Sections were analyzed with a Philips CM120 Electron Microscope (FEI Company, Eindhoven, The Netherlands) equipped with a digital camera (Keen View, SIS, Germany). Quantification of immunogold label was performed as described

previously [13] on randomly selected cell profiles from at least two distinct grids for each condition. Label for MHCII was considered to associate with the delimiting membrane when the gold particle was within 20 nm of the delimiting membrane and with no cross-sectioned ILV in the direct vicinity. For each condition >500 gold particles were assigned. Results are presented as a percentage of total label (mean ± SD of three independent measurements).

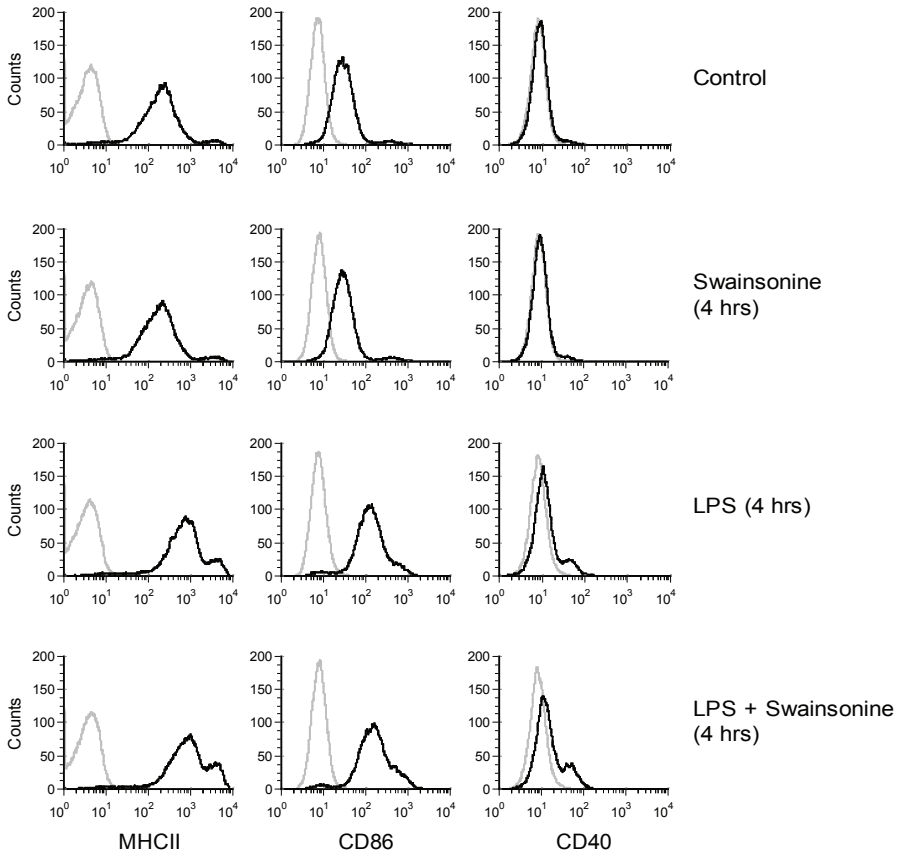
Acknowledgments

We thank M. de Boer-Brouwer for assistance with cell cultures and Dr E.N. Nolte-'t Hoen and Dr G. Raposo for helpful discussions.

Supplemental data



Supplemental figure 1. Distribution of MHCII in iDC versus mDC.
Representative electron microscopic views of cryosections of immature (control) and mature (24 hrs LPS) DC, immuno-gold labeled for MHCII. PM, plasma membrane; MVB, multi-vesicular body; M, mitochondrium; bars, 100 nm. Quantification of subcellular distributions is provided in Figure 1B.



Supplemental figure 2. No effect of swainsonine on the surface expression of MHCII, CD86 or CD40

Flow cytometric analysis of cell surface exposure of MHCII, CD86 and CD40 on DC that were cultured for 4 hrs in the absence or presence of LPS and/or swainsonine (black lines). Isotype controls are reflected by grey lines. Swainsonine did not influence cell surface expression of either of these markers in immature DC nor in LPS activated DC.

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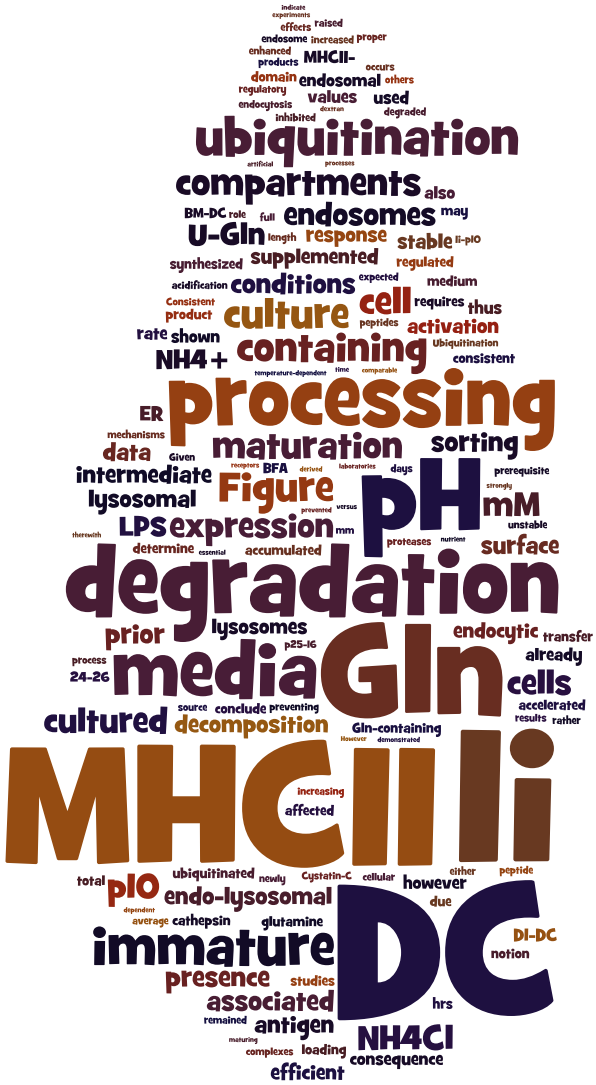
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Trafficking of MHC Class II in dendritic cells is dependent on but not regulated by degradation of its associated invariant chain

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Abstract

In dendritic cells (DC), newly synthesized MHCII is directed to endosomes by its associated invariant chain (Ii). Here, Ii is degraded after which MHCII is loaded with peptides. In immature DC, ubiquitination of peptide-loaded MHCII drives its sorting to lysosomes for degradation. Ubiquitination of MHCII is strongly reduced in response to inflammatory stimuli, resulting in increased expression of MHCII at the plasma membrane. Whether surface exposure of MHCII is also regulated during DC maturation by changing the rate of Ii degradation remained unresolved by conflicting results in the literature. We here pinpoint experimental problems that have contributed to these controversies and demonstrate that immature and mature DC degrade Ii equally efficient at proper culture conditions. Only when DC were cultured in glutamine containing media, endosome acidification and Ii degradation were restricted in immature DC and enhanced in response to lipopolysaccharide (LPS). These effects are caused by ammonia, a glutamine decomposition product. This artificial behavior could be prevented by culturing DC in media containing a stable dipeptide as glutamine source. We conclude that Ii degradation is a prerequisite for but not a rate limiting step in MHCII processing.

Introduction

Dendritic cells (DC) play a central role in initiating adaptive immune responses. Peripheral DC migrate in response to inflammatory cues to draining lymph nodes, where they can present peptides from peripherally sampled proteins in association with MHC class II (MHCII) to CD 4^+ T cells [1, 2]. Newly synthesized MHCII is associated in the endoplasmic reticulum (ER) with its chaperone invariant chain (Ii) [3]. The Ii-exoplasmic domain occludes the MHCII peptide binding groove, therewith preventing premature peptide loading of MHCII [4]. The Ii-cytoplasmic domain contains sorting information that is required for efficient transfer of MHCII out of the ER and its transport via the plasma membrane [5-8]

to endosomes [9-12]. Upon arrival of the MHCII–Ii complex at endosomes, Ii is degraded by endosomal proteases in a stepwise fashion [13, 14]. Cleavage of the intermediate Ii degradation product p10 by cathepsin S removes Ii-encoded sorting signals and allows subsequent antigen loading onto MHCII [15-18]. Effective antigen presentation to CD 4^+ T cells relies on stable cell surface expression of MHCII–peptide complexes, along with costimulatory receptors. We and others have shown that in immature DC, MHCII is ubiquitinated after Ii processing [19-22]. Ubiquitination of MHCII drives its incorporation into endocytic vesicles as well as its sorting into the luminal vesicles of multivesicular bodies [20, 21]. These sorting events ultimately result in the transfer to and degradation of MHCII in lysosomes [20, 21, 23]. Depending on the type of DC, ubiquitination of MHCII is ablated in response to a maturation stimulus [20, 21, 24-26], which has been attributed mainly to the developmentally regulated expression of ubiquitin E3-ligase MARCH I [24-26], therewith preventing its lysosomal degradation and allowing stable surface expression. Ubiquitin conjugation to MHCII thus seems to be the major regulatory event preventing stable MHCII surface expression by immature DC. Processing of associated Ii beyond the intermediate degradation product p10 is a prerequisite for ubiquitination of MHCII [21] and thus would be expected to occur efficiently already in immature DC. Some studies, however, claimed that processing of Ii-p10 is more efficient during DC maturation, driving both antigen loading and the removal of Ii-encoded endocytosis signals from MHCII, thus increasing surface expression of antigen loaded MHCII [27]. A correlative decrease in maturing DC of a cathepsin S inhibitor, Cystatin-C, was initially thought to regulate this process [27, 28]. Later studies showed, however, that Cystatin-C is localized at the trans-Golgi network (TGN) rather than at endosomal compartments [29] and that Ii processing was not affected in DC from Cystatin-C knock-out mice, disproving a regulatory role for Ii processing [30]. Furthermore, in a number of other laboratories, processing of Ii was not accelerated in maturing DC (reviewed in 31), leaving the controversy unexplained. We here report that in glutamine (Gln) containing culture media, immature DC fail to efficiently process MHCII associated Ii. Gln is an essential but unstable nutrient for cell culture that decomposes into pyrrolidone–carboxylic acid and the weak base ammonia (NH_3), which is protonated to NH_4^+ at physiological pH [32]. Because of this instability, Gln is often replaced by more stable di-peptides, such as

L-Alanyl-L-Glutamine as a Gln source. Our data indicate that the inhibitory effect of Gln on Ii processing was due to the pH neutralizing NH_4^+ that accumulates in endocytic compartments. At physiological conditions, Ii processing is already efficient in immature DC and not accelerated during DC maturation. We conclude that the processing of MHCII in DC is strictly regulated through its ubiquitination.

Results and discussion

Degradation of Ii is affected by the presence of Gln in cell culture medium

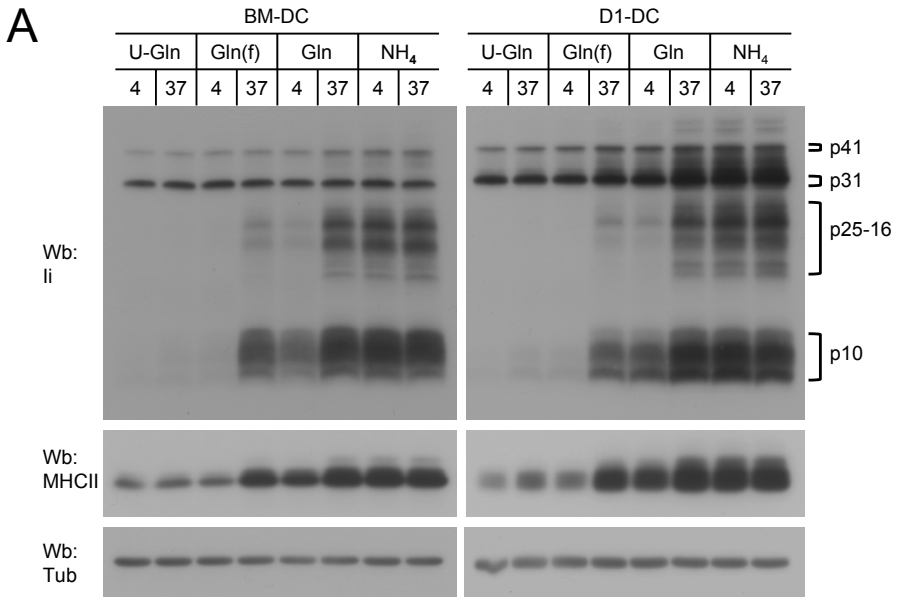
Gln is commonly added at 2-4 mM as an essential nutrient to cell culture media [33]. However, Gln is unstable in culture media at 37 °C and decomposes with a half-time of 2-4 days [32]. While the concentration of NH_4^+ in healthy tissues is below 0.1 mM [34], it is known that at concentrations above 1 mM, it elevates the pH of endocytic compartments. Gln derived NH_4^+ may thus interfere with the degradation of Ii by pH sensitive lysosomal proteases. This was tested for a DC cell line (D1-DC) and for freshly isolated bone marrow derived DC (BM-DC) (Figure 1). DC were first cultured in media containing the stable dipeptide L-Alanyl-L-Glutamine (UltraGlutamine-I, U-Gln) as a Gln source, and then transferred for 16 hrs to either U-Gln or 4 mM Gln-containing media. Both types of media are commonly used for tissue culture. The cells were then analyzed by Western blotting for the presence of Ii and its intermediate degradation products, using an antibody that recognizes the cytoplasmic domain of Ii. In comparison to DC that remained in U-Gln containing media, DC in Gln supplemented media accumulated more full length Ii p31 and p41 splice variants and much more of their intermediate degradation products p25-16 and p10. To test whether time and temperature-dependent decomposition of Gln plays a role, media were supplemented with fresh Gln and then stored for 4 days either at 4 °C or 37 °C prior to use for cell culture [lanes Gln(f) in Figure 1A]. Particularly prior storage at 37 °C interfered with Ii degradation. These effects were even more pronounced in media that were commercially supplemented with 4 mM Gln, where advanced Gln decomposition could be expected as a consequence of long-term storage (lanes Gln). Medium supplemented with 4

mM Gln, a concentration used for standard tissue culture, contains ~2 mM NH_4^+ after 2-4 days at 37 °C [32]. Consistent with this notion, U-Gln containing medium that was supplemented with 2 mM NH_4Cl indeed had comparable inhibitory effects on Ii processing. Both Gln and NH_4Cl also raised total cellular MHCII, while a control protein, tubulin, remained unaffected. This indicates a prolonged half-life of MHCII at these conditions, which is consistent with the notion that ubiquitination of MHCII, a prerequisite for its lysosomal targeting, requires prior degradation of its associated Ii [10, 35]. Together, these results indicate that time and temperature-dependent accumulation of NH_4^+ in Gln containing medium interfered with Ii processing in endosomes and with the degradation of MHCII in lysosomes. Given the comparable results for BM-DC and D1-DC, the following experiments are illustrated for D1-DC only, unless stated otherwise.

Endocytosis of MHCII-Ii complexes is driven mainly by di-leucine based endocytosis motifs in the cytoplasmic domain of Ii [10, 12, 35]. On immature DC, cell surface expression of MHCII was greatly reduced by the presence of Gln (threefold) or 2 mM NH4Cl (fivefold) (Figure 1B), despite of the increase in total cellular MHCII (Figure 1A). The net redistribution of MHCII to intracellular compartments at these conditions can be explained by the strong endocytosis motifs provided by associated full length Ii, p25-16 or p10. Surface expression of the DC activation marker CD86 (Figure 1B) and CD40 (not shown) was low at all conditions, demonstrating that neither Gln nor NH_4Cl influenced the DC maturation status.

The pH of the endo–lysosomal system is affected by Gln-containing media

Inefficient Ii degradation by immature DC in medium supplemented with Gln may be a consequence of artificially elevated pH values of MHCII-Ii carrying compartments. To determine the luminal pH of endocytic compartments, dextran conjugated to both fluorescein isothiocyanate (FITC) (a pH sensitive dye) and tetramethylrhodamine (TMR) (a pH insensitive dye) was endocytosed as a fluid phase marker for 2 hrs and chased for 30 min. Fluorescence intensities of FITC and TMR were then measured for individual compartments in live cells by confocal scanning laser microscopy. FITC/TMR fluorescence intensity ratios were related to pH by calibration after mild fixation and pH clamping of the cells (Supplemental figure). Dextran labeled endosomes/lysosomes in DC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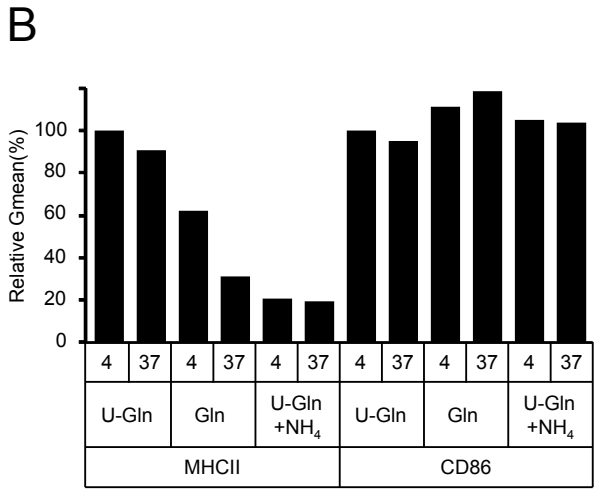
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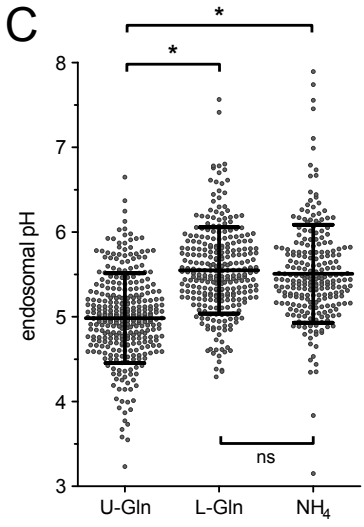
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grown in U-Gln containing media had a pH of 4.99 ± 0.53 (mean ± SD). The pH of endocytic compartments in cells cultured with Gln or both U-Gln and 2 mM NH4Cl was significantly raised to 5.55 ± 0.51 and 5.51 ± 0.58, respectively (Figure 1C). No gross morphological differences with respect to endosome size or distribution were observed in immature DC at the distinct culture conditions (data not shown). These data are consistent with the idea that interference with Ii degradation by Gln or NH_4Cl resulted from increasing endosomal pH. Cathepsin S, which is responsible for further processing of the intermediate Ii degradation product p10, has a broad pH optimum and is active at neutral pH [17, 36]. However, procathepsin S activation requires autocatalytic processing to cathepsin S, and this occurs only at low pH [36]. Given that Ii is processed prior to MHCII sorting in endosomes [21], cathepsin S activity is required in endosomes rather than lysosomes. It is possible that the rise in endosomal pH, due to Gln decomposition, interferes with the pH dependent activation of newly synthesized procathepsin S in endosomes.

Degradation of Ii-p10 is not enhanced during DC maturation

To determine the rate of Ii processing directly, we first tried ^{35}S-methionine pulse-chase experiments. Using such assays, however, it was impossible to evaluate Ii processing in immature DC as a consequence of polluting bacterial substances in commercially available ^{35}S-methionine that triggered DC

Figure 1 (left). Ammonia generated by Gln decomposition interferes with Ii processing by elevating the endosomal pH

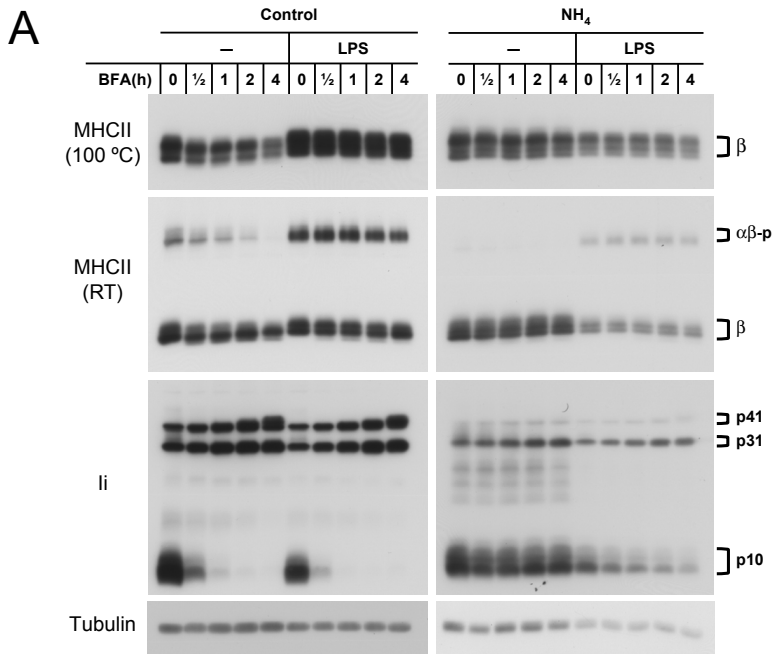
D1-DC and BM-DC were cultured for 16 hrs in media that were supplemented and stored before use for 4 days at 4 or 37 °C as indicated. U-Gln, control medium with ultraglutamine; Gln (f), medium with freshly added 4 mM Gln; Gln, medium purchased with Gln (4 mM) that had been stored at 4 °C for several months; NH_4Cl, medium supplemented with U-Gln and 2 mM NH_4Cl **(A)** Cell lysates were analyzed by western blot (Wb) for Ii, MHCII-β and tubulin (loading control) as indicated. The data shown are representative for three independent experiments. **(B)** Cell surface expression of MHCII and CD86 was determined by flow cytometry. The results shown are representative of three independent experiments. **(C)** D1-DC were allowed to endocytose FITC/TMR–dextran for 2 hrs, washed and chased for 30 min. Live cell images were acquired at 37 °C and ratiometric fluorescence analyses were performed as described in Materials and Methods and Figure S1. Each dot represents the pH of an endosomal/lysosomal compartment. Average values ± SD are given for each condition. Asterisk indicates significant differences with $p < 0.0001$; ns indicates nonsignificance with $p = 0.403$. p values were calculated by pairwise comparison using t-tests. The data shown are representative for three independent experiments.

maturation (data not shown). As an alternative approach, we used Brefeldin A (BFA) to block transfer of newly synthesized MHCII–Ii complexes out of the ER and measured the rate of degradation of Ii that had already egressed the ER prior to BFA addition (Figure 2). As expected, full length Ii (p31 and p41) accumulated in the presence of BFA due to the block in transfer to the degradation pathway. In contrast, p25-16 and p10 intermediate Ii degradation products, which already had been transported beyond the ER prior to the addition of BFA, were rapidly degraded, for p10 with an interpolated t½ of 20 min.

Although several laboratories find that processing of Ii occurs independently of the maturation status of DC [37-39], others reported that degradation of Ii is accelerated [27, 40, 41] and that the efficiencies of MHCII antigen loading are also increased [41-43], possibly as a consequence of a drop in lysosomal pH [40, 41]. The latter studies, however, were performed in Gln-containing media. We found that DC activation by lipopolysaccharide (LPS) did not change the rate of p10 degradation when DC were cultured with U-Gln. In the presence of 2 mM NH_4Cl (Figure 2) or Gln (data not shown), however, processing of p10 was strongly inhibited while additional LPS treatment resulted in intermediate processing rates of Ii-p10.

Others have used total cell measurements to determine average endo-lysosomal pH values and reported increasing lysosomal acidification during DC maturation [40, 41]. To examine potential pH changes in cells that were

Figure 2 (right). Ii degradation is not accelerated in maturing DC
D1-DC were cultured in U-Gln-containing medium and incubated for 16 hrs with or without LPS in the presence or absence of 2 mM NH_4Cl. BFA was either absent (0), present during the entire incubation (4), or present during the last 1/2, 1 or 2 hrs of incubation as indicated. **(A)** Cells were lysed in SDS sample buffer, either at room temperature to prevent dissociation of peptide-loaded MHCII complexes (αβ-p), or heated to 100 °C for full dissociation, then separated by SDS–PAGE and analyzed by western blotting for Ii, MHCII-β or tubulin. The blots shown are representative for three independent experiments. **(B)** Quantification of signals for p10 relative to the signal at t = 0 (set at 100% for each condition) as shown in the western blot for Ii in (A). Values were normalized according to tubulin signals in cell lysates. **(C)** BM-DC or D1-DC were cultured for 2 hrs with FITC/TMR–dextran in the presence or absence of LPS, washed and chased for 16 hrs in the indicated media. pH measurements were performed as in Figure 1C. Calculated pH values: BM-DC U-Gln (5.20 ± 0.19), NH_4 (6.04 ± 0.31), LPS (4.92 ± 0.42), LPS + NH_4 (5.96 ± 0.32), D1DC U-Gln (4.60 ± 0.76), NH_4 (6.26 ± 0.10), LPS (5.32 ± 0.23), LPS + NH_4 (6.23 ± 0.38). Asterisk indicates significant differences from the control U-Gln sample with $p < 0.05$ analyzed by one-way anova followed by a Dunnet multiple comparison test; ns indicates nonsignificance.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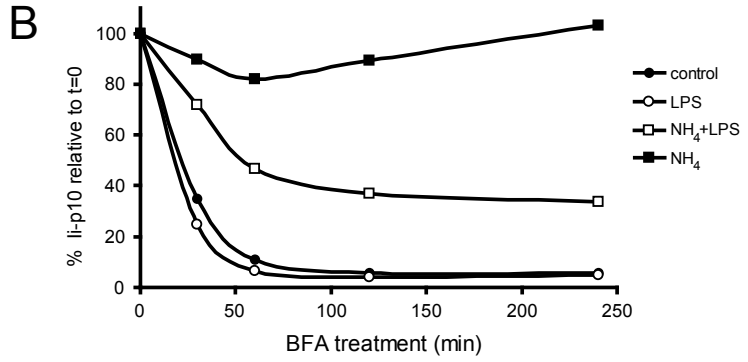
B
% Ii-p10 relative to t=0
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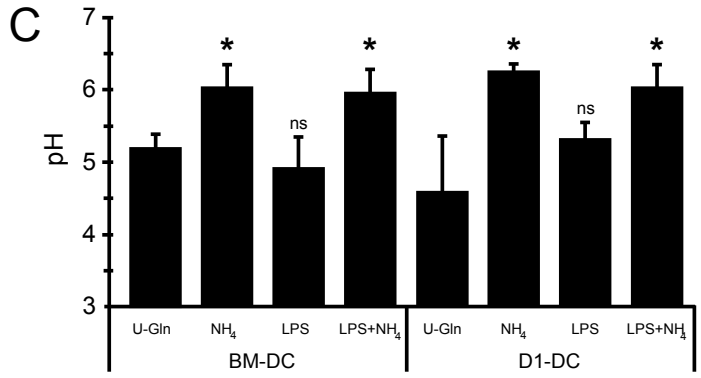
C
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BM-DC
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cultured in U-Gln-containing media, we determined the pH values of individual endo-lysosomal compartments. D1-DC or BM-DC were pulse-labeled for 2 hrs with fluorescently labeled dextran and chased for 16 hrs in the presence or absence of LPS (Figure 2C). In the absence of NH_4Cl, no significant changes in the average endo-lysosomal pH values were observed in response to LPS (in BM-DC 5.20 ± 0.20 versus 4.92 ± 0.43 and in D1-DC 4.60 ± 0.76 versus 5.33 ± 0.23). Consistent with our previous data, the presence of 2 mM NH_4Cl significantly increased the pH values of endo-lysosomal compartments. Also at this condition, LPS treatment had no significant effect on the average endo-lysosomal pH. We thus conclude that the pH of endo-lysosomal compartments is not modulated during DC activation.

Ubiquitination of MHCII is inhibited when DC are cultured in Gln-containing media

We and others have previously demonstrated that sorting of MHCII to lysosomes is driven by ubiquitination of its β-chain [19-22, 24-26]. Only peptide-loaded MHCII is ubiquitinated and interference with Ii degradation by protease inhibitors blocked ubiquitination, indicating that ubiquitination of MHCII-β requires prior degradation of associated Ii [21]. Given that NH_4^+ from decomposed Gln prevented p10 degradation, we anticipated that also ubiquitination of MHCII would be compromised at these culture conditions. To determine ubiquitination of MHCII, MHCII was immunoprecipitated from cell lysates and subsequently analyzed for MHCII-β, Ii and ubiquitinated proteins by western blotting (Figure 3). The immunoprecipitated samples contain less p31/p41 when compared with total cell lysates due to the relatively large pool of non-MHCII-associated Ii in the ER. Again, Ii degradation intermediates accumulated in the Gln and NH_4Cl-treated immature DC but not in mature DC. Consistent with previous observations, ubiquitination of MHCII-β was prominent in immature DC but less efficient in LPS activated DC, when cells were cultured in U-Gln-containing media (Figure 3). In contrast, only little MHCII-β was ubiquitinated in immature DC when cultured in the presence of either 2 mM NH_4Cl or Gln, consistent with the notion that ubiquitination of MHCII-β requires prior degradation of its associated Ii.

In summary, we conclude that proteolytic processing of Ii is inhibited when DC are cultured in Gln-containing media. NH_4^+ accumulated in the media

as a consequence of Gln decomposition and raised the pH of endosomes, reducing the activity of pH dependent lysosomal proteases that process Ii. Only at proper culture conditions, where DC are cultured with U-Gln instead of Gln, Ii processing is already efficient in immature DC and not accelerated during DC maturation. Our findings explain the controversy in the literature regarding enhanced Ii processing in response to LPS maturation, by demonstrating that this occurs only upon artificial modulation of endo-lysosomal pH.

As demonstrated in Figure 3, our findings have implications for the analyses of the mechanisms regulating MHCII ubiquitination. For example, studies on the regulatory mechanisms of MHCII ubiquitination, e.g. MARCH I expression [24-26], may be difficult to reproduce or interpret when Gln containing media are used. In general, processes that rely on proper endosome acidification, such as the generation of peptides from endocytosed antigens, antigen cross-presentation [44], activation or inactivation of signaling receptors [45] may be affected by Gln decomposition. MHCII has been instrumental to study

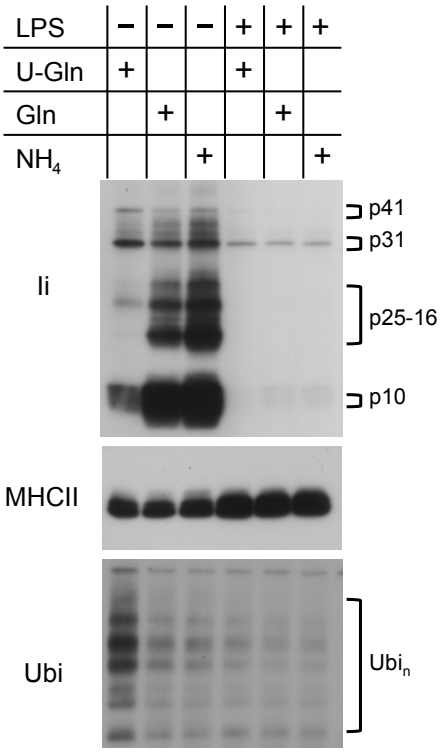


Figure 3. Ubiquitination of MHCII is inefficient in DC that are cultured in Gln-containing media
D1-DC were cultured in the presence or absence of LPS for 24 hrs in media containing Gln, NH_4Cl or U-Gln as indicated. MHCII was immunoprecipitated from cell lysates, eluted at 100 °C and analyzed by western blotting for MHCII-β, Ii and ubiquitin. Ub_n indicates oligoubiquitinated MHCII-β. The blots shown are representative for three independent experiments.

general mechanisms of endosomal sorting because of its processing along the endocytic pathway. The decomposition of Gln is likely to affect a wide range of other cellular processes, in DC or other cell types, that, like Ii processing, also rely on properly acidified endosomes or lysosomes. In conclusion, our data demonstrate that unstable Gln containing media should be avoided when studying antigen presentation by DC and are consistent with the notion that MHCII surface expression is regulated by ubiquitination of MHCII rather than by Ii processing.

Materials and Methods

Reagents

Brefeldin A (Penicillium brefeldianum) was obtained from Sigma–Aldrich and used at 10 µg/ml. Rabbit polyclonal antibodies directed against cytoplasmic domains of mouse MHCII-β and Ii [21] were kindly provided by Dr N. Barois. Ubiquitinated MHCII-β was detected with horseradish peroxidase conjugated anti-ubiquitin antibody P4D1 (Santa Cruz Biotechnology, Inc.) as described previously [21]. Anti-α-tubulin clone TUB-1A2 was purchased from Sigma–Aldrich.

Cell culture

BM-DC were isolated as described in Ref. [46] from C57BL/6 mice. The dendritic cell line, D1-DC [47], and BM-DC were maintained in Iscove's Modified Dulbecco's medium supplemented with 2 mM ultraglutamine 1 (both from Biowhittaker), 10% heat inactivated FCS (Sigma), 100 IU/ml penicillin and 100 mg/mL streptomycin (both from GIBCO), 50 µm β-mercaptoethanol and 30% conditioned medium from GM-CSF producing NIH 3T3 cells (R1) [47]. For DC maturation, cells were incubated for 18–26 hrs with LPS (Escherichia coli, serotype 0111:B4 from Fluka) at 10 µg/ml.

Western blotting and immunoprecipitation

D1-DC or BM-DC were lysed on ice in 1% Triton-X100, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10 mM N-ethylmaleimide (Sigma–Aldrich) and complete protease inhibitor mix

(Roche). After removal of nuclei by centrifugation, lysates were either directly used for western blotting according to standard techniques or first processed for immunoprecipitation and then western blotted. For immunoprecipitations, rat anti-mouse I-A/I-E monoclonal antibody (M5/114.15.2, obtained from BioLegend) was added, incubated for 1 hr at 4 °C and precipitated with Protein G agarose beads (Pierce). Beads were washed and eluted in SDS sample buffer for 5 min at 100 °C. Western blot signals for Ii and tubulin were quantified using Supersignal® West Pico Chemiluminescent Substrate (Pierce), the ChemiDoc™ XRS luminescent image capture system and quantity one software (Bio-Rad).

Flow cytometric analysis

DC were harvested in PBS containing 2 mM EDTA and stained with Allophycocyanin-conjugated anti-mouse I-A/I-E monoclonal antibody M5/114.15.2 (eBioscience) or FITC-conjugated anti-mouse CD86 (B7-2) monoclonal antibody GL1 (Becton Dickinson–Pharmingen) for 30 min on ice in PBS containing 1% BSA. Control cells were stained with the corresponding isotype control antibodies. After washing, cells were measured using an FACSCalibur™ (Becton Dickenson) and analyzed using FCS Express (De Novo Software).

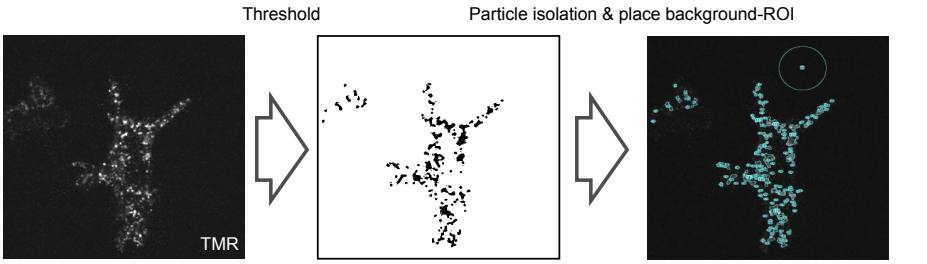
pH measurements of endocytic compartments

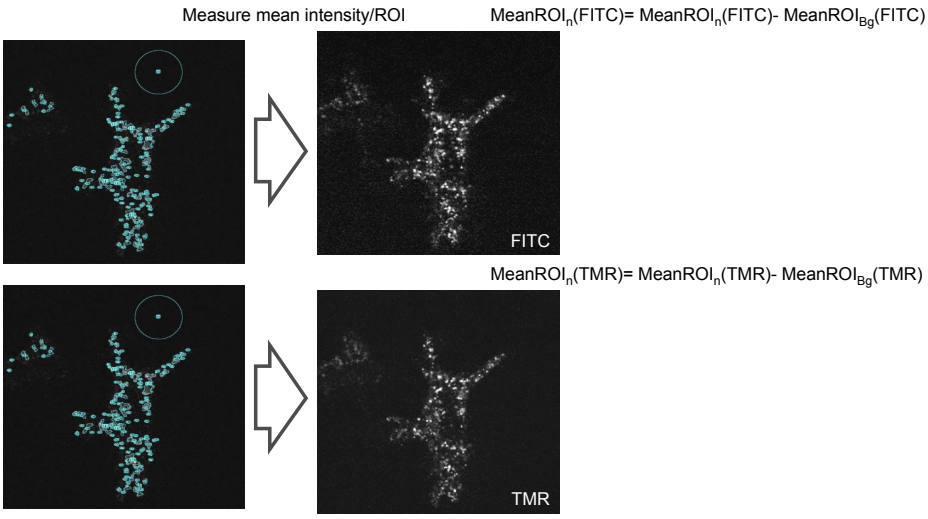
Amino-dextran (MW70) was conjugated with FITC and TMR succinimidyl esters (all from Invitrogen) as described in Ref. [48]. Cells were incubated for 2 hrs in the continuous presence of labeled dextran, washed and chased for 30 min. For LPS treatments, dextran was internalized during the first 2 hrs of LPS stimulation and image acquisition was performed after overnight culture. BM-DC were identically pulsed with the dextrans in culture dishes and cells were replated onto fluorodishes after two washes in culture media. Again, imaging was performed after overnight culture. Each set of experiments was followed by calibration measurements in 0.1 M KPO4 buffers ranging from pH 6.8 to 4.5 after fixation in 2% paraformaldehyde for 20 min. Endosomes were defined and FITC/TMR signal ratios within endosomes were determined using ImageJ (Dr W. Rasband, US National Institutes of Health, Bethesda) with plugins from Dr G. Landini (University of Birmingham, UK) as described in Figure S1. Sigmoid curve fitting of ratios in pH-clamped samples was performed in GraphPad Prism 5 (GraphPad Software) and recalculated in Excel 2007 (Microsoft Corporation).

Acknowledgments

We acknowledge Dr N. Barois for providing antibodies, M. de Boer-Brouwer for assistance with cell cultures, Dr C. van der Lest for advice on statistical analyses and Dr E.N. Nolte-'t Hoen for helpful discussions and critically reading the manuscript.

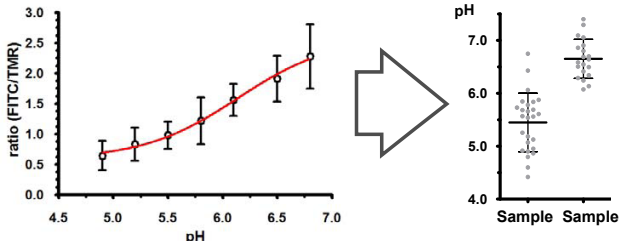
Supplemental data





RatioROI$_n$ = (MeanROI$_n$(FITC))/(Mean ROI$_n$(TMR))

Sigmoid curve fit (pH=-log(T-B)/(ratio-B)-1)-log(EC$_{50}$) => transform sample ratios to pH
T=top of range; B=bottom of range; EC$_{50}$=reversal point



Supplemental figure (left). pH measurement of endocytic compartments
D1-DC were grown in 10-mm well fluorodishes (WPI Ltd) for 2 days after which FITC/TMR–dextran (5 mg/ml) was internalized for 2 hrs. Labeled cells were washed four times and chased either for 30 min or overnight. BM-DC were pulsed with the FITC/TMR dextrans for 2 hrs in the culture dish. Cells were then harvested, replated onto fluorodishes and imaged after overnight culture. Cells were transferred to a climatized microscope chamber (37 °C, 5% CO_2) and allowed to equilibrate for 30 min before images of at least 10 separate fields per sample were acquired for the FITC and TMR labels (ex 488/em 515/30, ex 568 nm/em 590LP). Fixation was performed by adding one volume of freshly prepared 4% paraformaldehyde (Aurion) to the media for 20 min. Fixative was removed by extensive washing and cells were placed in 130 mM KPO4-buffered solutions with pH ranging from 6.8 to 4.5 for 10 min before calibration images were acquired (at least 10 fields for each calibration pH value). TMR labeled endocytic structures were identified and isolated by thresholding. Within each image background intensities were determined in a region outside the cells. For isolated regions of interest (ROIs) mean intensities in the original FITC and TMR images were determined and subjected to local background subtraction. A minimal remaining intensity of 3 was chosen as threshold. Ratios between FITC and TMR were determined for individual ROIs. Values from calibration images were used to correlate pH values to FITC/TMR ratios by sigmoidal fitting [pH = –(log (T –B)/(ratio – B) – 1 – log (EC 50), in which T = top of curve, B = bottom of curve and EC50 is reversal point]. The variables were used to convert ratios observed in the ROIs of live cell images. Plots and statistical analyses were performed in Graphpad Prism 5 (La Jolla).

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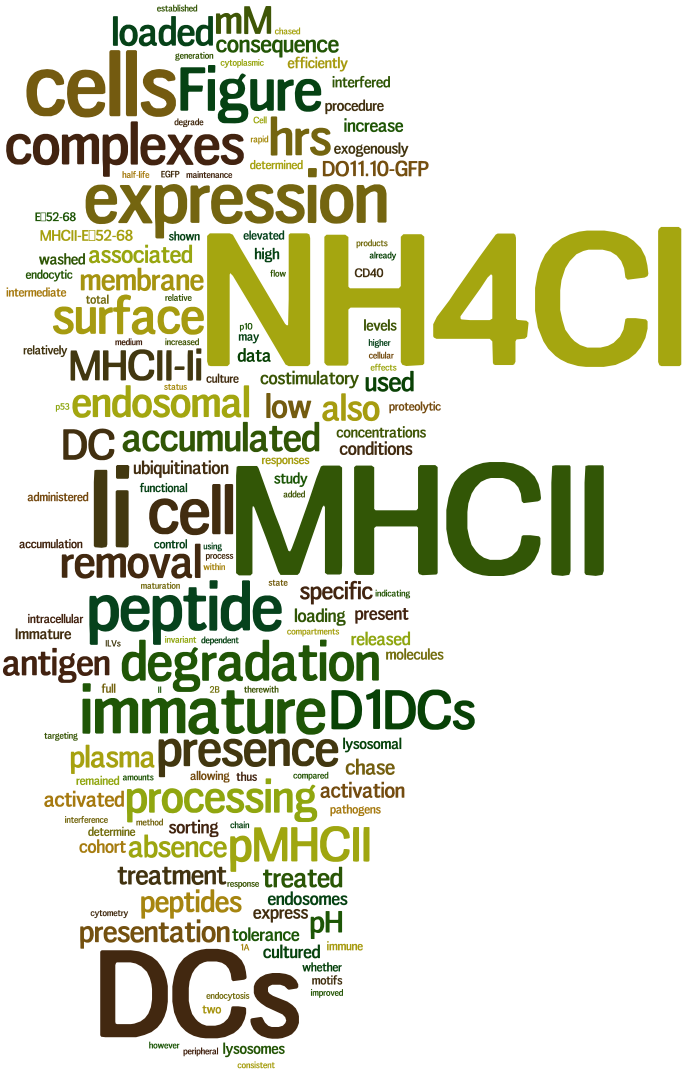
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6

A method to enhance MHC class II mediated antigen presentation by immature dendritic cells

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To be submitted

Abstract

Presentation of peptides by MHC class II (MHCII) on dendritic cells (DCs) may either activate or tolerize $CD4^+$ T cells, depending on the status of the DC and the T cell. Immature DC are potent inducers of T cell tolerance. These DC express relatively little MHCII as a consequence of ubiquitination driven transport to and degradation of MHCII in lysosomes. Proteolytic removal of MHCII-associated invariant chain (Ii) occurs in endosomes and is a prerequisite for both MHCII peptide loading and its ubiquitination. Addition of 2 mM NH_4Cl to DC culture media elevated the endosomal/lysosomal pH and hence interfered with Ii degradation by pH dependent lysosomal proteases. We used this condition to artificially accumulate a pool of MHCII-Ii complexes in immature DCs. After NH_4Cl removal, degradation of Ii rapidly resumed and accumulated intracellular MHCII became available for peptide loading and temporal cell surface expression. This procedure did not affect the immature status of DCs as concluded from the unaltered expression of transcriptionally regulated CD40. Eventually this pool of MHCII was ubiquitinated and degraded in lysosomes, recovering the normal level of MHCII surface expression on immature DCs. MHCII that was released from the NH_4Cl block could be efficiently loaded by exogenously added peptides and such specific complexes were temporarily highly expressed at the immature DC plasma membrane. This procedure improved the ability of immature DCs to present pMHCII to T cells and could potentially be used for the development of novel therapeutic DC-based strategies for directing immune tolerance.

Introduction

Conventional dendritic cells (DCs) function in the initiation of adaptive immune responses as well as in maintenance of self tolerance. At steady state conditions, DCs are present in peripheral tissues in a non-activated or immature state, at which they sample their environment using various endocytic mechanisms,

and monitor the presence of pathogens. To prime adaptive immune responses against pathogens, DCs need to be activated by conserved pathogen associated molecular patterns (PAMPs)[1]. PAMPs are sensed by DCs through pattern recognition receptors (PRRs), which include the family of Toll-like receptors (TLRs). In response to these ”danger” signals, DCs enter a differentiation process or “maturation” program [2], which includes an upregulation of the expression of costimulatory molecules and inflammatory cytokines. In addition, maturing DCs transiently increase their endocytic capacity, allowing efficient uptake and intracellular proteolytic processing of pathogens [3-6]. Immature DCs constitutively express MHCII molecules which are loaded within endosomes with locally present self peptides. Immature DCs do, however, not efficiently express peptide loaded MHCII (pMHCII) at their plasma membrane as a consequence of intracellular sorting to and degradation in lysosomes. Activated DCs on the other hand temporarily increase the synthesis of MHCII and, most importantly, increase MHCII surface expression at the post-translational level through interference with its targeting to lysosomes. Thus, while immature DCs generally express low amounts of pMHCII, mature DCs express high amounts of pMHCII. Activated DCs are boosted to migrate from peripheral tissues towards the secondary lymphoid organs where they may present their acquired pathogen-derived peptides on MHCII to naïve $CD4^+$ T cells expressing the T cell receptor (TCR) specific for these pMHCII complexes, resulting in the initiation of antigen specific immune responses. DCs do however already have constitutive migratory properties at their immature state, and antigen presentation by immature DC functions in establishing and maintaining peripheral tolerance by a variety of mechanisms. These include deletion or induction of anergy of antigen specific T cells, and stimulation of the proliferation and differentiation of regulatory T cells (Treg) [7-10]. At least three types of signals determine the functional outcome of T cell responses, TCR interactions with pMHCII, (absence or presence of) interactions between costimulatory molecules, and cytokine signaling [11].

Newly synthesized MHCII is associated with the invariant chain (Ii) in the endoplasmatic reticulum [12]. Sorting information encoded in the cytoplasmic domain of this chaperone guides the complex via the Golgi to the endocytic system [13], but also prevents premature peptide loading of MHCII by occupying

its peptide-binding groove [14]. At endosomes, Ii is removed from MHCII by progressive proteolytic trimming, and cleavage of the intermediate Ii degradation product, p10, liberates MHCII from the cytoplasmic sorting motifs present in Ii [15, 16]. Finally, only a small Ii fragment, called CLIP (class II associated invariant chain peptide), remains associated with the peptide binding groove until it is exchanged for other peptides present within the endosome, in a process that is facilitated by another chaperone called DM [17]. Many of the hydrolases that are responsible for the generation of peptides from endocytosed pathogens, and those that degrade MHCII associated Ii, are dependent on a relatively low endosomal pH. Experimental elevation of the endosomal pH by adding weak bases (e.g. chloroquine or NH_4Cl), interferes with the degradation of full length Ii and its intermediate degradation products [18, 19]. Besides for peptide loading, removal of the Ii also leaves MHCII susceptible for ubiquitination [20], a post-translational modification that in immature DCs drives the efficient sorting of MHCII to the intraluminal vesicles (ILVs) of a late endosomal compartment that is also referred to as the multi-vesicular body (MVB). This pathway drives the transfer of peptide loaded MHCII to lysosomes and its degradation therein, and is the major determinant in the relative short half-life of MHCII in immature DCs [20, 21]. Part of the pMHCII complexes escape immediate sorting to ILVs after their generation at endosomes, thus allowing immature DCs to express moderate levels of pMHCII at their plasma membrane. Immature DCs also actively endocytose pMHCII, allowing a new opportunity for ubiquitin-driven sorting at MVBs, thus ensuring maintenance of low MHCII surface expression levels. Such low expression of surface MHCII, which in principle are loaded with self-peptides due to absence of foreign antigens, may however be functional in maintaining tolerance. Conventional DCs that are activated by inflammatory stimuli cease to ubiquitinate MHCII, resulting in high and stable surface expression of MHCII [20-23].

In a previous study we demonstrated that the presence of glutamine or low concentrations of NH_4Cl in culture medium raised the pH of endosomal compartments, and therewith interfered with endosomal degradation of MHCII associated Ii [18, 19]. Because removal of Ii from MHCII is a prerequisite for its ubiquitination and thus also for sorting to ILVs [20], lysosomal targeting and degradation of MHCII was inhibited at these conditions. Furthermore, we observed that upon removal of NH_4Cl, Ii processing was resumed and followed

by synchronous peptide loading of the cohort of previously accumulated MHCII and transport to the cell surface. This resulted in relative high expression levels of pMHCII at the plasma membrane of immature DC. In the current study we set out to investigate whether this artificial procedure could be used to improve antigen presentation by and cognate T cell interaction with immature DCs.

Results

NH_4Cl reduces Ii degradation and accumulates MHCII intracellularly

Lysosomal proteases require a mildly acidic endosomal environment to efficiently degrade MHCII associated Ii. Addition of the weak base NH_4Cl elevated the pH of endosomal compartments and as a consequence interfered with Ii processing [18, 19]. To determine the dose-response of this effect, the murine DC cell line D1 (D1DC) was cultured for 18 hrs in the absence or presence of increasing amounts of NH_4Cl (0-10 mM) and changes in total cellular MHCII and Ii were analyzed by Western blotting (Figure 1A). NH_4Cl dramatically interfered with Ii processing in a dose dependent manner, as evidenced by the accumulation of the full length p31 and p41 splice variants of Ii, but also of the intermediate Ii degradation products p25 to p10. As a consequence, MHCII ubiquitination was severely reduced [18-20] in comparison to non-treated cells. Despite the higher total cellular content of MHCII (Figure 1A), its expression at the plasma membrane was reduced up to 5-fold at 10 mM, as determined by flow cytometry (Figure 1B,C). Thus, NH_4Cl exposure resulted in an intracellular accumulation of MHCII-Ii complexes. This can be explained by the presence of two di-leucine motifs encoded in the cytoplasmic tail of the Ii [24-26] that drive rapid endocytosis. As a result, MHCII-Ii complexes mainly reside intracellularly. Cell surface expression of the costimulatory molecules CD40 (Figure 2B) and CD86 remained low at these conditions (see also [18, 19]), demonstrating that NH_4Cl did not induce DC maturation.

Release of NH_4Cl accumulated MHCII

Next, we investigated whether the processing of accumulated MHCII-Ii complexes in immature DCs could be restored by simply removing NH_4Cl from

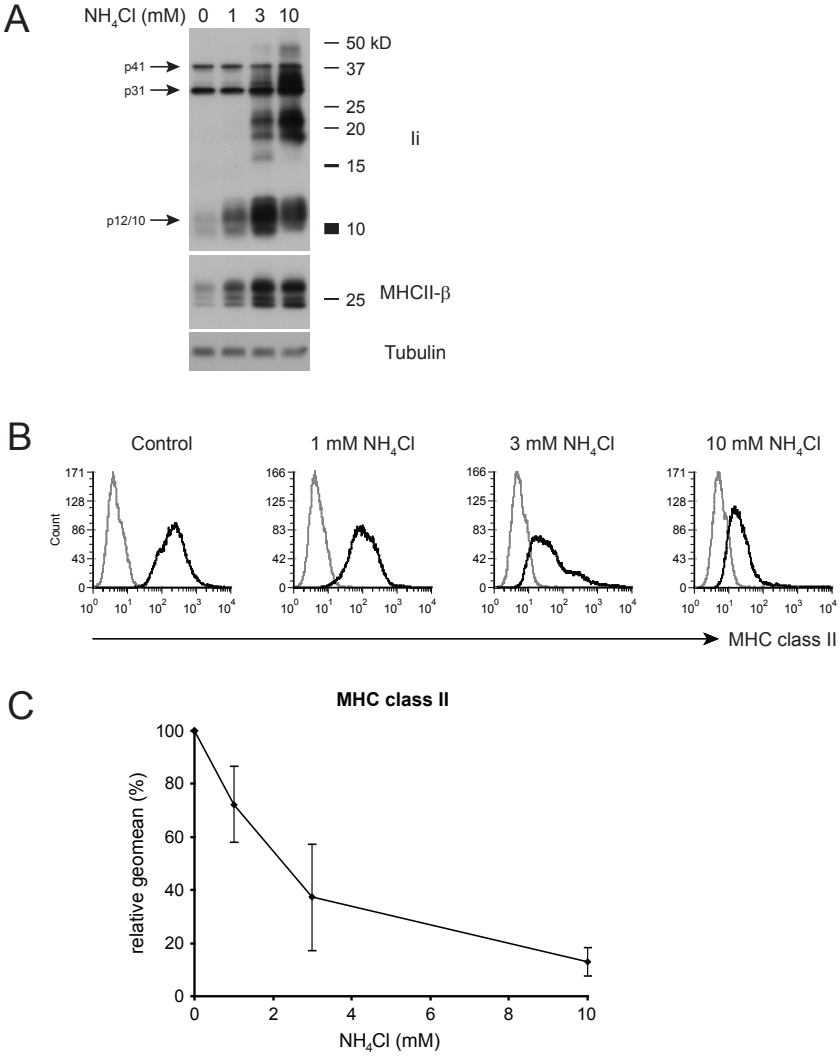

Figure 1. Changes in MHCII processing and cell surface expression induced by NH_4Cl
(A) D1DCs were cultured for 18 hrs with 0, 1, 3 or 10 mM NH_4Cl as indicated, and analyzed by Western blotting for Ii, MHCII-β and tubulin (as a loading control) as indicated. Full length Ii splice variants p41 and p31 and partial Ii degradation products (p12/10) are indicated. Other intermediate Ii degradation products are also visible between 15 and 25 kD. Samples were lysed in SDS containing buffer and boiled at non-reducing conditions. **(B)** D1DCs were treated as in (A) after which cell surface expression of MHCII was determined by flow cytometry (black line). Isotype controls are plotted in gray. Figures are representative of at least three independent experiments. **(C)** Cell surface expression of MHCII was quantified as in (B). The geomean of untreated cells was set to 100%, and data are expressed relative to this value. Figure represents the average (±SD) of three independent experiments.

the culture medium. Hereto, we first cultured D1DCs for 18 hrs in the absence or presence of 2 mM NH_4Cl. This concentration was previously established to mildly elevate endosomal pH [18], and already induced a profound accumulation of MHCII-Ii (Figure 1) but did not provoke major morphological changes or alter the overall presence of endosomal marker proteins on MHC II positive compartments as determined by fluorescence microscopy (data not shown). D1DCs were then washed and chased in the absence of NH_4Cl. The endosomal pH was rapidly restored from 5,3±0,07 to 4,3±0,08 within one minute after NH_4Cl removal (Supplementary figure). Recovery of the endosomal pH reinstated proteolytic activity necessary for Ii degradation as seen by the rapid processing of previously accumulated Ii products (Figure 2A). Control samples in which the chase was performed in the presence of 2 mM NH_4Cl showed no change in Ii processing. After a 3 hrs chase in the absence of NH_4Cl, the amount of full length Ii (p41 and p31) approximated control levels in D1DCs that had not been exposed to NH_4Cl. During the chase there was a clear but transient increase of the intermediate Ii degradation product p10 with a peak at 60 min, consistent with synchronized processing of a cohort of accumulated Ii. MHCII that was accumulated during the NH_4Cl treatment largely persisted during the 3 hrs chase after NH_4Cl removal, indicating a relatively long half-life after degradation of associated Ii. Cell surface expression of MHCII increased almost four fold between 1-3 hrs chase after NH_4Cl removal, which was even two fold higher compared to control cells that were not treated with NH_4Cl (Figure 2B). The kinetics of MHCII appearance at the plasma membrane is consistent with that of the Ii processing after removal of NH_4Cl. The expression of MHCII at the plasma membrane of 18 hrs NH_4Cl treated and 3 hrs chased D1DCs was comparable to that of 3 hrs LPS activated D1DCs. In contrast to LPS treated D1DCs, the maturation status of NH_4Cl treated D1DCs remained unaffected during the chase, as indicated by maintenance of a low cell surface expression of the costimulatory molecule CD40 (Figure 2B). Together these data show that the cohort of MHCII-Ii complexes that was build up in NH_4Cl treated D1DCs, was synchronously released for further processing upon NH_4Cl removal.

NH_4Cl treatment enhances surface expression of specific pMHCII complexes

We next studied whether MHCII in NH_4Cl accumulated MHCII-Ii complexes

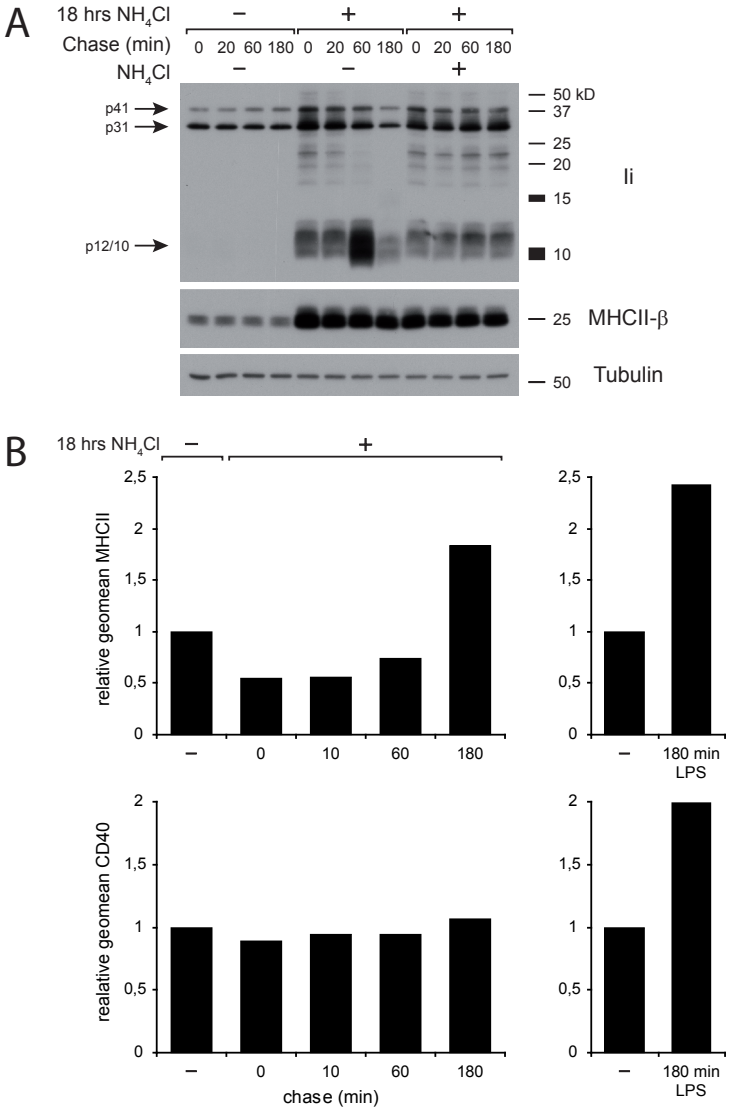

Figure 2. Processing of accumulated MHCII-Ii complexes after NH_4Cl removal
(A) D1DCs were cultured for 18 hrs in the absence or presence of 2 mM NH_4Cl, washed and chased for 0, 20, 60 or 180 min in the absence or presence of NH_4Cl as indicated. Cells were lysed and analyzed by immunoblotting for Ii, MHCII and tubulin (loading control). Full length Ii splice variants p41 and p31 and partial Ii degradation products (p12/10) are indicated at the left, MW markers at the right. **(B)** Immature D1DCs were treated as in (A) and cell surface expression of MHCII (top panel) and CD40 (lower panel) were determined by flow cytometry. The geomean of untreated cells (-) was set to 1, and values for NH_4Cl treated cells that were chased for 0, 10, 60 or 180 min in the absence of NH_4Cl are expressed relative to this value. Figures are representatives of at least three independent experiments. As comparison, non NH_4Cl treated cells are compared with 3 hrs LPS activated cells on the right.

could be loaded with exogenously added peptides after release from this block. Hereto, D1DCs were pre-treated for 18 hrs with 2 mM NH_4Cl, washed and chased for 3 hrs in the absence of NH_4Cl and presence of different concentrations of a peptide corresponding to amino acids 52-68 of the I-Eα protein. The monoclonal antibody Y-Ae was then used to determine cell surface expression of $E\alpha_{52-68}$ loaded MHCII complexes (MHCII-$E\alpha_{52-68}$) [27] (Figure 3). Y-Ae labeling of the plasma membrane was significantly increased after prior accumulation of MHCII-Ii by NH_4Cl (Figure 3A). MHCII-$E\alpha_{52-68}$ could clearly be detected for NH_4Cl treated D1DCs at a peptide concentration of 1 μM, but not for non NH_4Cl treated cells (Figure 3B), indicating sensitization of the $E\alpha_{52-68}$ presentation process. Analogous to D1DCs, also bone marrow derived DCs (BMDCs) displayed a dramatic increase of both total MHCII and MHCII-$E\alpha_{52-68}$ complexes when peptides were loaded after NH_4Cl treatment (Figure 3C). The signal for MHCII-$E\alpha_{52-68}$ complexes in control cells stabilized after 2 hrs in the presence of $E\alpha_{52-68}$ peptide, probably as a consequence of the equilibrium that is reached between formation and degradation of pMHCII in immature DCs. We conclude that the accumulated cohort of MHCII-Ii that is released upon NH_4Cl removal can be efficiently loaded with exogenously administered peptide to increase cell surface expression of specific pMHCII complexes by immature DCs.

NH_4Cl treatment increases the efficiency of antigen presentation to T cells

To determine whether NH_4Cl treatment could enhance the efficiency of antigen presentation to T cells we used the OVA-specific murine T cell hybridoma DO11.10-GFP. This T cell hybridoma is stably transfected with a construct encoding for EGFP controlled by the nuclear factor of activated T cells (NFAT)-responsive promoter [28] and is rather independent of costimulation for its activation. Although this T cell line is generated from BALB/c mice, and thus is I-A^d restricted, it is also stimulated by I-A^b when loaded with a peptide corresponding to amino acids 323-339 from ovalbumin ($OVA_{323-339}$ peptide) [29, 30]. Activated DO11.10-GFP cells produce EGFP, which can easily be detected by flow cytometry.

D1DCs were cultured for 18 hrs in the absence or presence of 2 mM NH_4Cl, washed and then cultured for 3 hrs in the absence of NH_4Cl and the presence of the $OVA_{323-339}$ peptide. Control cells were washed and cultured

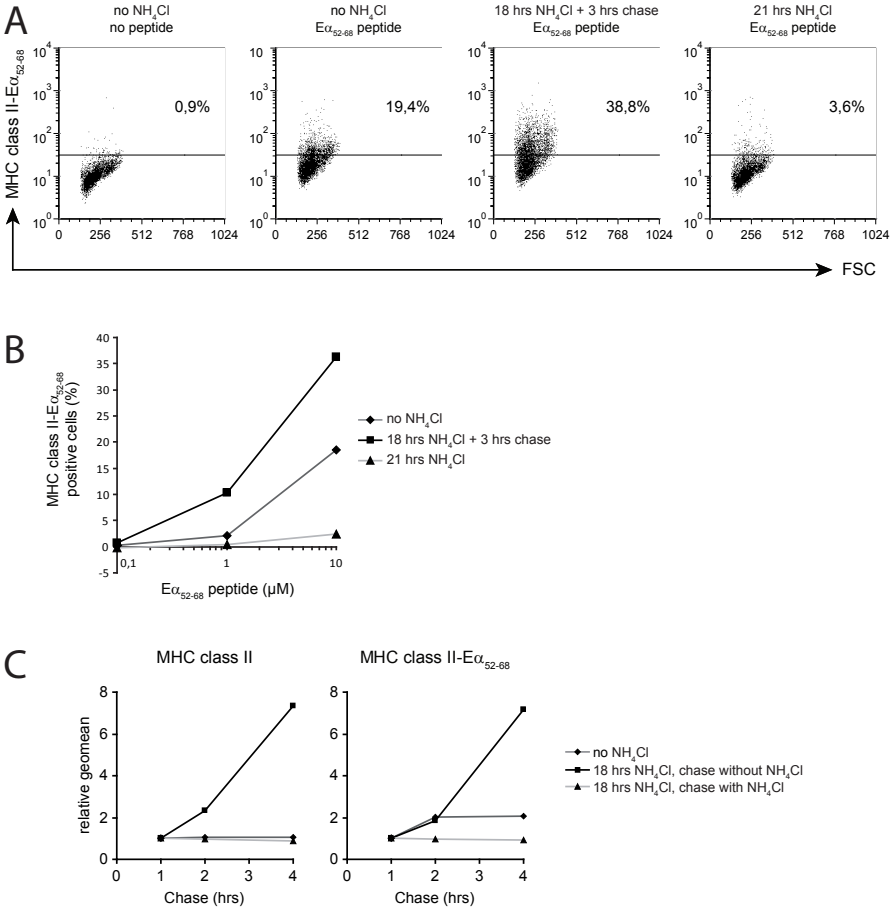

Figure 3. NH_4Cl treatment of DCs increases cell surface expression of peptide loaded MHCII

(A) Immature D1DCs were cultured for 18 hrs with 2 mM NH_4Cl. Control cells were left untreated (no NH_4Cl). D1DCs were chased for 3 hrs with (21 hrs NH_4Cl) or without NH_4Cl (18 hrs NH_4Cl + 3 hrs chase) in the absence (no peptide) or presence of 10 μM $E\alpha_{52-68}$ peptide. Cell surface expression of MHCII-$E\alpha_{52-68}$ was determined by flow cytometry using Y-Ae antibody and is plotted against forward scatter (FSC). Indicated percentages represent the percentage of MHCII-$E\alpha_{52-68}$ positive cells as defined by their position above the indicated threshold line determined by cells that were cultured in the absence of peptide and labeled with Y-Ae. **(B)** Summarizing graph of the percentage of MHCII-$E\alpha_{52-68}$ positive cells as determined in (A) for different peptide concentrations. Signals are corrected for background which has been obtained from parallel D1DCs that had not received peptide. Figures are representatives of three independent experiments. **(C)** Immature BMDCs were treated as in A) but chased for 1, 2, or 4 hrs in the presence or absence of 1,0 μM $E\alpha_{52-68}$ peptide. Total MHCII and MHCII-$E\alpha_{52-68}$ was analyzed by flow cytometry. The geomean labeling intensity of cells chased for 1 hr was set to 1, and data are expressed relative to this value. Figures are representatives of at least three independent experiments.

in the presence of peptide and 2 mM NH_4Cl. D1DCs were then allowed to interact with DO11.10-GFP cells and EGFP expression was determined by flow cytometry (Figure 4). Cocultured DO11.10-GFP cells and D1DCs were identified separately by their expression of respectively CD4 and CD11c (Figure 4A). Gated DO11.10-GFP cells were plotted for the percentage of cells that expressed GFP above background (Figure 4B). When 10 μM $OVA_{323-339}$ peptide was administered to the culture medium, activation of the DO11.10-GFP cells improved approximately three fold when D1DCs had received NH_4Cl treatment (Figure 4C). As can be expected, continuous presence of NH_4Cl negatively affected activation of DO11.10-GFP cells.

We verified these effects in an independent assay using a p53 peptide specific T cell clone (p53 T cells) [31]. In this assay single T cells were discriminated by their expression of CD4 and low FSC ($CD4^{high}FSC^{low}$) from the D1DCs ($CD4^{low}$) and D1DC-T cell conjugates ($CD4^{high}FSC^{high}$) in the coculture (Figure 5A). Activation of p53 T cells was determined by their IFN-γ expression. Again, D1DCs pre-treated with NH_4Cl where superior in stimulating T cells (Figure 5B,C). In summary, we conclude that the accumulated cohort of MHCII-Ii that is released upon NH_4Cl removal can be efficiently loaded with exogenously administered peptides and is able to sensitize antigen presentation of these peptides to specific T cells.

Discussion

In this study we demonstrate that antigen presentation by immature DC can be enhanced by a simple experimental regimen that increases the amount of pMHCII at the DC surface. Newly synthesized MHCII-Ii complexes were first accumulated in the endocytic tract in the presence of NH_4Cl and then released by removing NH_4Cl. The cohort of released MHCII could be loaded with exogenously added peptide and expressed at the cell surface for at least 8 hrs (Figures 4,5 and [19]). This procedure significantly elevated pMHCII at the plasma membrane of immature DCs, therewith increasing the cognate interaction with T cells, as demonstrated for two distinct T cell clones. Immature DCs continuously synthesize and degrade MHCII with a short half-life of ~2 hrs.

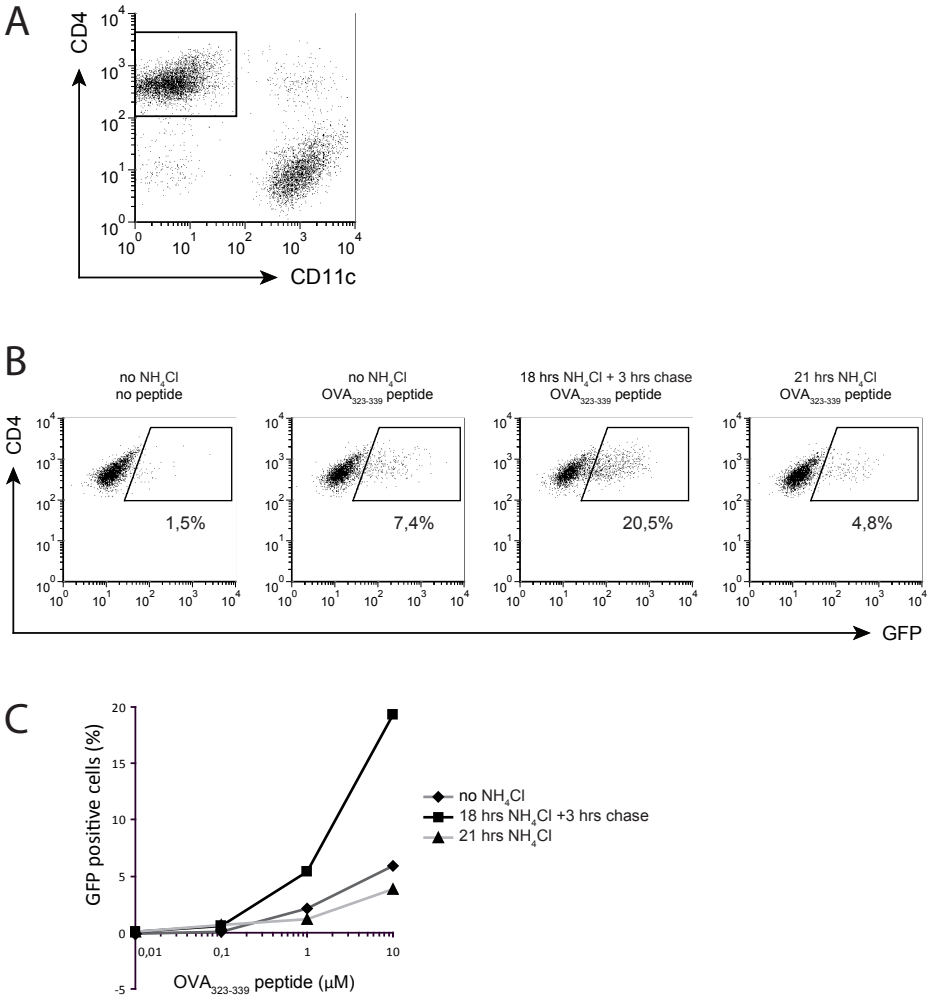

Figure 4. Enhanced presentation of $OVA_{323-339}$ peptide after NH_4Cl treatment
MHCII-Ii complexes were accumulated in immature D1DCs for 18 hrs in the presence of 2 mM NH_4Cl. Control cells were left untreated (no NH_4Cl). D1DCs were chased for 3 hrs with (21 hrs NH_4Cl) or without NH_4Cl (18 hrs NH_4Cl + 3 hrs chase) in the absence (no peptide) or presence of indicated concentrations of $OVA_{323-339}$ peptide. DO11.10-GFP cells were then added in a 1:1 ratio and after 24 hrs coculture analyzed by flow cytometry for GFP expression. **(A)** Dot-plot of cocultured D1DCs and DO11.10-GFP cells stained for CD11c and CD4. The $CD4^+/CD11c^-$ DO11.10-GFP cells were gated as exemplified by the indicated rectangle. **(B)** Plots of gated DO11.10-GFP cells (from cells with 10 μM peptide) according to their expression of CD4 and GFP. Percentages of GFP positive cells are indicated in each plot, as defined by the indicated gates. **(C)** Percentages of GFP-expressing DO11.10-GFP cells as exemplified in (B). Signals are corrected for background signals obtained from non-peptide loaded cells at otherwise identical conditions. Figures are representative of at least three independent experiments.

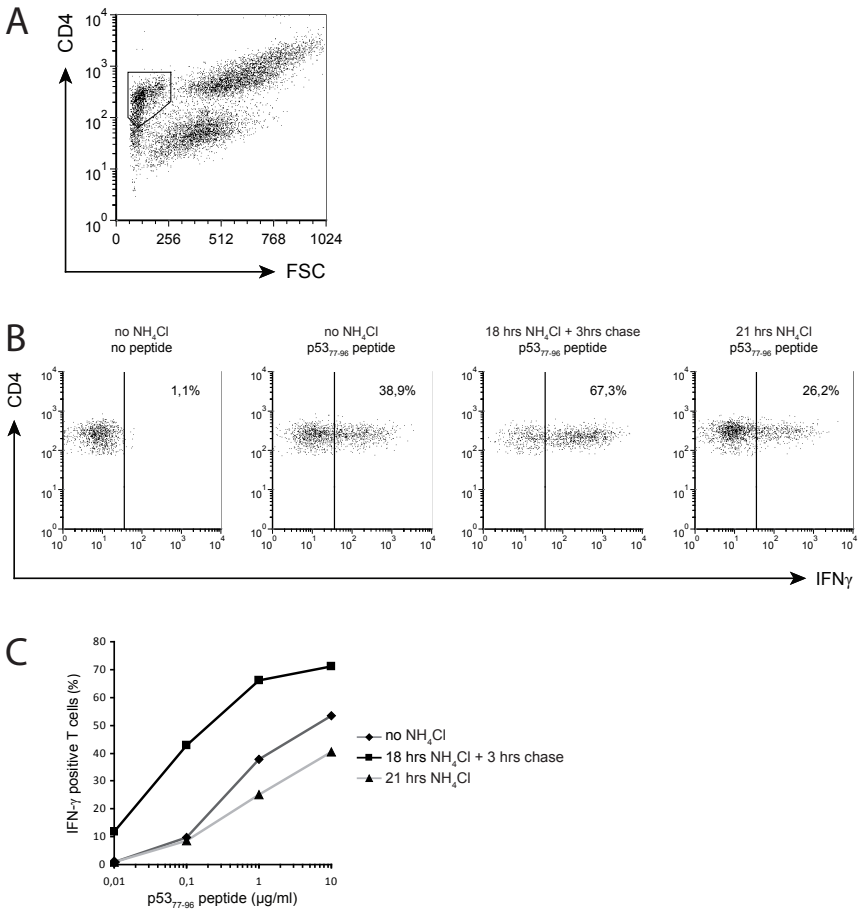

Figure 5. Enhanced presentation of $p53_{77-96}$ peptide after NH_4Cl treatment
MHCII-Ii complexes were accumulated in immature D1DC for 18 hrs in the presence of 2 mM NH_4Cl. Control cells were left untreated with NH_4Cl (no NH_4Cl). D1DC were chased for 3 hrs with (21 hrs NH_4Cl) or without NH_4Cl (18 hrs NH_4Cl + 3 hrs chase) in the absence (no peptide) or presence of indicated concentrations of $p53_{77-96}$ peptide. p53-specific T cells were then added and cells were allowed to interact for 6 hrs. T cells were analyzed for IFN-γ expression by flow cytometry. **(A)** Dot plot showing cocultured D1DC and p53-specific T cells clone stained for CD4. The gate indicates single $CD4^+$ T cells and was used for further analysis. Dots with high CD4 expression and high FSC represent clusters of interacting D1DC and T cells. **(B)** Plots of gated T cells from experiments in which 1,0 µg/ml $p53_{77-96}$ peptide was used, according to their CD4 and IFN-γ expression. Percentages of IFN-γ positive cells are indicated in each plot, as defined by the indicated threshold line as determined for cells that were cultured in the absence of peptide labeled for IFN-γ. **(C)** Percentages of IFN-γ-expressing T cells as exemplified in (B). Signals are corrected for background signals obtained from non-peptide loaded cells at otherwise identical conditions. Figures are representative of at least three independent experiments.

Therefore, the positive effect of the NH_4Cl treatment on antigen presentation is only transient. Indeed, when NH_4Cl-treated immature DCs were washed and cultured for another 24 hrs in the absence of NH_4Cl and the presence of $E\alpha_{52-68}$ peptide, we observed that surface expression of both total MHCII and MHCII-$E\alpha_{52-68}$ complexes had returned to control levels (data not shown).

To accumulate MHCII-Ii complexes we used 2 mM NH_4Cl, a physiological compound that displays little or no toxicity [32]. In the body NH_4^+ concentrations are below 0,1 mM at non-pathological conditions [33]. When administered at 2 mM, endosomal pH was only slightly elevated [18], but sufficient to interfere with endosomal Ii processing and lysosomal targeting and degradation of MHCII (Figure 1A), consistent with previous observations [18]. Cell growth and morphology did neither change notably, nor were DCs activated by this treatment, while NH_4Cl removal allowed rapid and full recovery of Ii processing (Figure 2A). We also tried higher concentrations (10 mM) of NH_4Cl, but the recovery of Ii processing upon NH_4Cl removal was much slower at these conditions (data not shown). Possibly, such high NH_4^+ concentrations may have interfered with the make-up of the endosomal system, or with the delivery of lysosomal hydrolases from the trans-Golgi network. By using 2 mM for 18 hrs, we achieved maximal effects without disturbing vital cellular processes. NH_4Cl-treated DCs accumulated MHCII-Ii complexes at the delimiting membranes of endosomes [19], as a consequence of the continued association of Ii-encoded endocytosis motifs with MHCII and interference with MHCII ubiquitination. NH_4Cl removal initiated synchronous elimination of Ii from endosomally accumulated MHCII-Ii complexes. The resumption of Ii processing at these conditions corroborates other data on the proteolytic capacity of endosomes in such treated DCs [19]. Proteolytic processing of the Ii allows peptide loading of MHCII and removes Ii-encoded endocytosis motifs from MHCII, therewith allowing relatively stable expression of pMHCII at the plasma membrane.

The described method improved antigen presentation and activation of two T cell clones. The D1DCs used in our study were not exposed to PAMPs and had low expression of costimulatory molecules at their surface: transcriptionally regulated CD40 remained low during the entire prodedure, indicating that the DC were not activated (see also [19]). CD86 slightly increased during chase after

NH_4Cl treatment, possibly as a consequence of interference with lysosomal degradation in the presence of ammonium, but remained low as compared to LPS activated DCs (data not shown). The costimulatory molecule CD80 was not measured in our studies. The functional consequence of T cell activation by the procedure as developed in this study remains unclear and requires further investigation. First, the effects of transient NH_4Cl incubations on primary DCs should be tested as well as the type of response by both naïve and antigen experienced T cells. The functional outcome of the T cell response, activation versus anergy induction, could be established by determining T cell proliferation and cytokine production upon subsequent activation by antigen-pulsed DC [34]. In addition, the possible tolerogenic potential of DC treated with our "NH_4Cl method" should be compared with established methods for in vitro generation of tolerogenic DCs [8] and should be investigated *in vivo*.

In vitro loaded DCs are already used in experimental clinical settings with the aim to improve vaccination strategies [35]. Vaccines based on immature DCs are also used to promote tolerance, for instance in *in vivo* models of autoimmune diseases [36, 37]. The simplicity and potency of our NH_4Cl method to transiently boost antigen presentation by immature DCs might prove to be helpful in optimizing the efficacy of such DC-based vaccines. Other advantages for the use of NH_4Cl in such therapies are its relative harmless nature and its high diffusion rate, making it safe for use in patients.

Materials and Methods

Cell culture

BMDCs were isolated from C57BL/6 mice and maintained as described [19]. Non-adherent cells were harvested and reseeded at day 7 and the assay was performed at day 9 of the culture. Quality of the BMDC culture was tested for cell surface expression of CD11c, MHCII, CD86 (B7-2) and CD40. The culture contained ≥95% DCs according to the expression of CD11c and ≤5% mature cells determined by CD86 and CD40 expression. The dendritic cell line, D1 [38], was maintained as described [18]. For maturation, D1DCs were stimulated wit 10 µg/ml LPS (E. coli, serotype 0111:B4 from Fluka). The OVA-specific I-Ad

restricted murine T cell hybridoma DO11.10-GFP, stably transfected with a construct encoding for eGFP controlled by the nuclear factor of activated T cells (NFAT)-responsive promoter [28], was kindly provided by Prof. Dr. A. Rudensky. DO11.10-GFP cells were maintained in RPMI+Glutamax (Gibco) supplemented with, 10% heat inactivated serum (Sigma), 1 mg/ml G418 (Geneticin, Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin. The p53 specific $CD4^+$ T cell clone (p53 T cells) [31], generated in a C57BL/6 p53-/- mouse, was kindly provided by Prof. Dr. C. Melief (Leiden University Medical Center, Leiden, The Netherlands). The p53 T cells recognize the murine p53 77-96 peptide ($p53_{77-96}$) and were maintained as described [39].

NH_4Cl treatment and antigen presentation assays

For NH_4Cl treatment, cells were cultured for 18 hrs in medium containing 2 mM NH_4Cl, unless other concentrations are stated. For chase conditions, cells were washed with and recultured in prewarmed medium with or without 2 mM NH_4Cl at 37 ºC, 5% CO_2. Peptide was added during the chase when indicated. After 3 hrs chase, T cells were added to D1DCs in a 1:1 ratio and cocultured for the time indicated. For analysis of IFN-γ production by p53 T cells, 10 µg/ml Brefeldin A (Sigma-Aldrich) was added for the last 2 hrs of culturing to accumulate IFN-γ at the secretory pathway prior to flow cytometric analysis and cells were fixed and permeabilized before staining the intracellular protein. The $E\alpha_{52-68}$, $OVA_{323-339}$ and the $p53_{77-96}$ peptides were manufactured by Genscript.

Endosomal pH measurements

D1DCs were cultured for 16 hrs in the presence of 2 mM NH_4Cl. To label late endocytic compartments the cells were cultured for another 2 hrs with dual labeled (FITC/TMR) dextran, washed and chased for 30 min in the continuous presence of NH_4Cl. While monitoring, NH_4Cl-treated D1DCs were washed three times with media lacking NH_4Cl. To determine the pH of endosomes/lysosomes in live cells, ratio imaging of FITC/TMR for individual compartments was performed on a MP2100 Biorad imaging station (Zeiss, Hemel Hempstead, UK) as described previously [18]. A customized algorithm to isolate labeled endosomes from the TMR channel was written in Cell Profiler 40 and used on live recordings of dextran labeled compartments to calculate fluorescence intensity ratios

within these regions. The removal of NH_4Cl was recorded on the microscope while maintaining a 37 ºC, 5% CO_2 culture environment. Data were processed in Excel (Microsoft, Redwood, USA), ratio conversion to pH was performed according to the calibration curves obtained in fixed samples as described [18]. Curve fitting and statistical analyses of pH datasets were performed in Graphpad Prism 5 (La Jolla, USA).

Western blotting

D1DCs were lysed on ice in buffer containing 1% Triton-X100, 1 mM EDTA pH 8, 20 mM Tris/HCl, 150 mM NaCl, 10 mM N-Ethylmaleimide (Sigma-Aldrich) and complete protease inhibitor mix (Roche). Nuclei were removed by centrifugation for 5 min at 13.000x*g*. Alternatively, D1DCs were lysed at room temperature in buffer containing 2% SDS, 62,5 mM Tris/HCl pH6,8 and 10% glycerol, passed five times through a 25G needle and centrifuged for 2 min at 13.000 x *g*. All samples were heated for 5 min at 100 ºC at non-reducing conditions and proteins were separated by SDS-PAGE and transferred to PVDF-membrane (0,2 µm pore size, Millipore) according to standard techniques. Ii and MHC class II-β were detected by rabbit polyclonal antibodies directed against their cytoplasmic domains, kindly provided by Dr N. Barois. Equal cell load was checked using anti-α-tubulin clone TUB-1A2 (Sigma-Aldrich). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Pierce Biotechnology Inc. Immunoblot signals were detected on film using Supersignal® West Pico Chemiluminescent Substrate (Pierce).

Flow cytometry

Cells were detached and harvested on ice in PBS containing 2 mM EDTA and stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated anti-I-A/I-E (M5/114.15.2), anti-IFN-γ (XMG1.2), anti-CD4 (L3T4) from eBiosciences, anti-CD40 (1C10) from Southern Biotechnology and anti-CD11c (N418) from Biolegend in PBS containing 1% BSA. For analysis of the specific MHC class II-E$\alpha_{52\text{-}68}$ complex, unconjugated anti I-A^b/E$\alpha_{52\text{-}68}$ (Y-Ae) from Santa Cruz and PE-conjugated goat anti-mouse IgG from BD Pharmingen were used. Non-specific staining was determined with corresponding isotype control antibodies. Intracellular IFN-γ was stained by first

fixing the cells in PBS/4% paraformaldehyde and permeabilisation in PBS/1% BSA containing 0,1% saponin. To correct for background of the I-A^b/$E\alpha_{52-68}$ staining, D1DCs that were not loaded with $E\alpha_{52-68}$ peptide were used, except for figure 3C. Cells were analyzed using a FACSCalibur™ (Becton Dickenson) and FCS Express software (De Novo Software).

Acknowledgments

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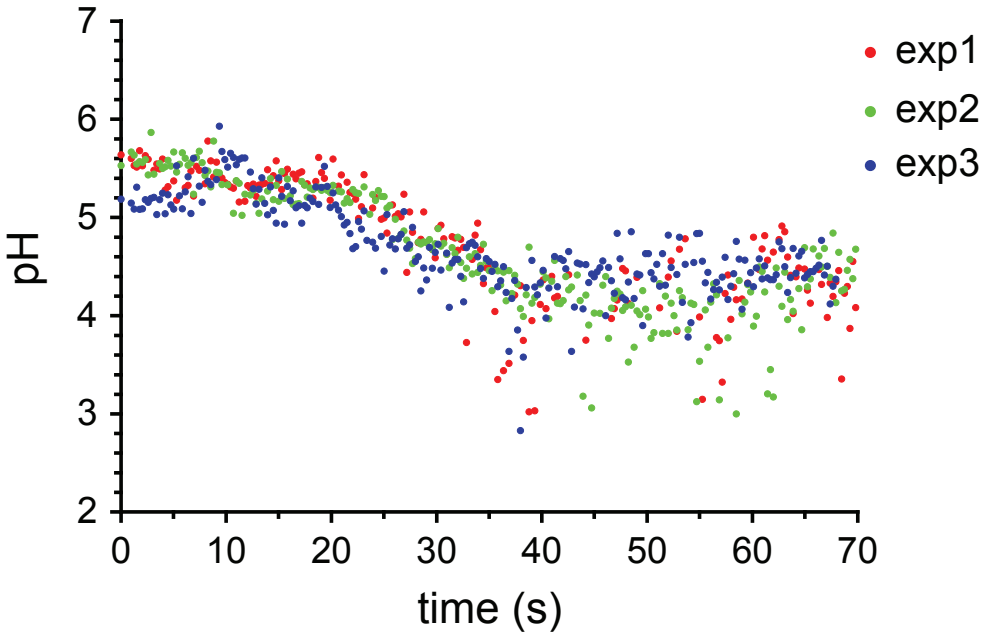
Supplemental data



Supplementary figure. Rapid recovery of endosomal pH upon removal of NH_4Cl
Ratio imaging of endocytosed dual labeled (FITC/TMR) dextran was used to determine endosomal pH in living cells. Cells were incubated with dextran for 2 hrs, washed with media containing NH_4Cl and chased for another 30 minutes on the microscope. NH_4Cl was removed during imaging by washing three times with NH_4Cl lacking medium. Recorded images showed a decrease in pH of individual endosomal compartments from an average pH of 5,3±0,07 to 4,3±0,08 within one minute after NH_4Cl removal (±SD, n=3, p<0,0001). Three representative ratio recordings are shown, color coded.

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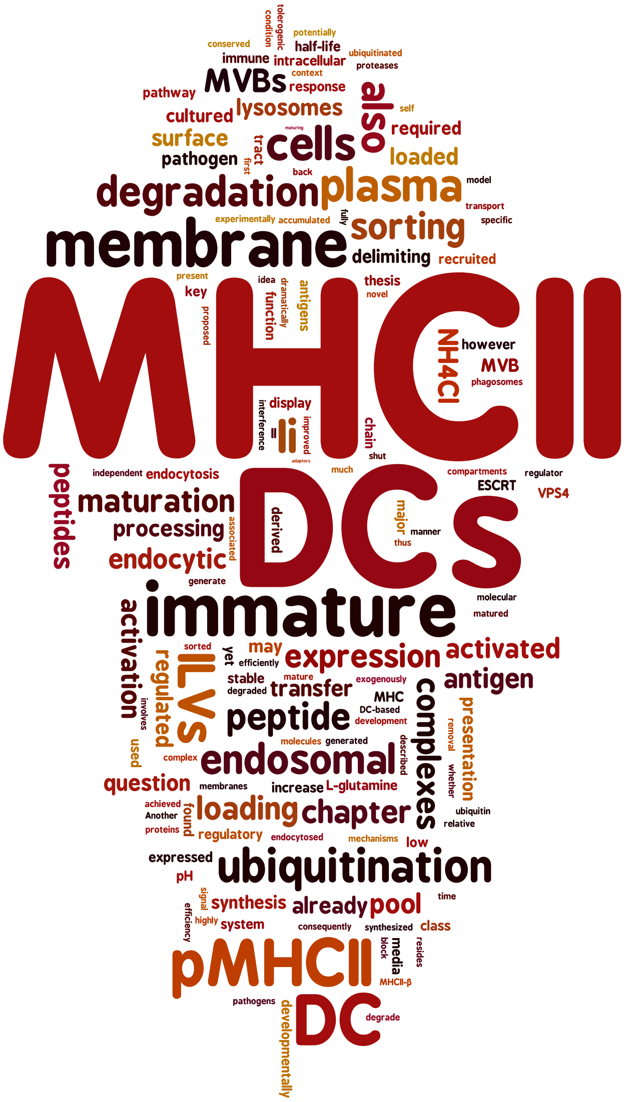
MHCII
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MVBs
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also
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expression
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7

Summarizing discussion

Summarizing discussion

Dendritic cells (DCs) are professional antigen presenting cells that constitutively express and renew peptide loaded major histocompatibility complex class II (pMHCII) at their plasma membrane to inform T cells of recently encountered "self peptides" and pathogen derived antigens. DCs that are activated by pathogens dramatically increase their display of pMHCII complexes, including those carrying peptides from endocytosed and proteolytically processed pathogens. The increase of pMHCII in response to DC activation is achieved through 1) a temporal increase followed by shut down of MHCII synthesis, and 2) interference with lysosomal degradation of MHCII. Together, these mechanisms provide a "snap-shot" of antigens that are present at the time of DC activation. Activated DCs then carry this information to secondary lymphoid organs for evaluation by T cells.

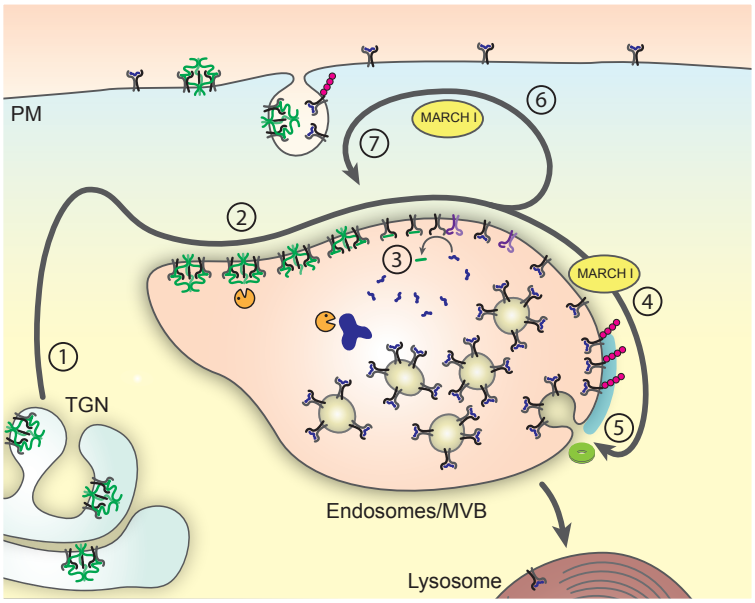
Activation of antigen specific naïve T cells receptors is an early and crucial event at the onset of an adaptive immune response. Whether the DC signal to T cells is immunogenic or tolerogenic is determined not only by the quality and quantity of the antigens that are presented on MHCII, but also by other regulatory cues such as the expression of costimulatory molecules and cytokine secretion. Immature DCs synthesize, process and degrade pMHCII complexes in a constitutive manner. Although MHCII is already expressed at non-inflammatory conditions by immature DCs, it largely resides within the endocytic tract at this condition and only at relatively low levels at the plasma membrane (see figure). MHCII in immature DCs has a short half-life (~2 hrs) which is sustained by transfer to and degradation in lysosomes. When DCs are activated by conserved pathogen associated molecular patterns, they generate a much more stable population of surface exposed pMHCII and concomitantly lose their endosomal/lysosomal pool. The molecular mechanism behind this redistribution and stabilization of MHCII during DC maturation is the topic of this thesis.

The synthesis of MHCII increases upon DC activation, but this is only transient and synthesis is completely abrogated in fully matured DCs. Yet, surface expression of MHCII is maximized for fully matured DCs. This already

indicates that the major determinant for pMHCII expression lies in the rate of its degradation rather than in its biosynthesis. In **chapter 2** of this thesis we demonstrate that the degradation of MHCII in immature DCs depends on ubiquitination of the MHCII-β chain. After its synthesis in the endoplasmic reticulum, MHCII is first transported to the endocytic tract with the aid of invariant chain (Ii), a chaperone for MHCII . After arriving in the endocytic tract, Ii is degraded by endosomal proteases, liberating MHCII and making it susceptible for peptide loading. Degradation of MHCII associated Ii is also a prerequisite for MHCII ubiquitination. Only in immature DCs, oligoubiquitin chains are covalently bound to a highly conserved unique lysine in the cytoplasmic domain of the MHCII-β chain. This ubiquitin tag is used to sort MHCII at multi vesicular bodies (MVBs) for transfer to lysosomes. This pathway involves incorporation of MHCII into the intra luminal vesicles (ILVs) of MVB. Some pMHCII may escape a first round of sorting at MVBs and consequently appear at the plasma membrane of immature DCs. These complexes can then be endocytosed for a second opportunity of being sorted at MVB to the ILV. Aside its sorting at MVB, also the endocytic uptake of pMHCII appears to be facilitated by its ubiquitination. In activated DCs, ubiquitination of MHCII ceases, and hence its active sorting to lysosomes, resulting in stable cell surface expression. Our results described in chapter 2, together with the identification by others of MARCH I as the major E3 ubiquitin ligase for MHCII, have greatly improved our understanding of how MHCII surface expression is developmentally regulated in DCs. Targeting of MHCII ubiquitination in DCs might be a good strategy for the development of novel DC-based immune therapies.

Transfer of MHCII to ILVs may also occur, although much less efficient and probably by default, in a ubiquitination independent manner. Such a pathway may however contribute to MHCII processing. Another open question concerned the relative contributions of ubiquitination driven endocytosis and sorting at MVBs in the determination of the MHCII half-life in immature DCs. Sorting of ubiquitinated transmembrane proteins at MVBs involves the endosomal sorting complex required for transport (ESCRT). In contrast, endocytosis of ubiquitinated membrane proteins is regulated through other adaptors. Vacuolar protein sorting 4 (VPS4) is a key regulator of ESCRT function and also required for the actual pinching of ILVs. In **chapter 3** we found that expression of a

immature DC



MHCII-Ii

MHCII-CLIP

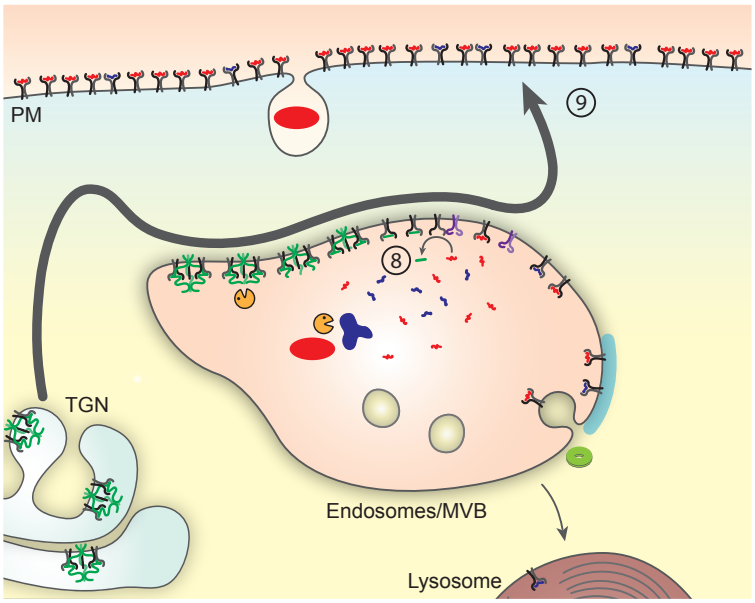
MHCII-self peptide

MHCII-pathogen peptide

ubiquitinated MHCII

DM

activated DC



proteases

self-protein

pathogen

VPS4

ESCRT

dominant-negative mutant of VPS4 did not influence the relative distribution of MHC class II between surface and intracellular compartments, indicating that shuttling of MHCII between the plasma membrane and the endosomal system is independent of ESCRT function. Dominant-negative VPS4 did however significantly prolong the half-life of MHCII in immature DCs, underscoring the importance of MVB sorting in MHCII homeostasis. The efficiency of peptide loading of MHCII was unaffected, supporting the idea that transfer of MHCII to ILVs solely serves to irreversibly transfer MHCII to lysosomes for degradation and is not required for productive antigen loading.

At equilibrium in immature DCs, approximately 50% of MHCII resides in ILVs of the MVBs. One model proposed that this intracellular "storage pool" could be recruited in activated DCs through back fusion of ILVs with the endosomal delimiting membrane. In another model it was proposed that MHCII on mature DCs was primarily synthesized during DC maturation, leaving previously generated pMHCII complexes at MVBs to be degraded in lysosomes. The pool of MHCII at ILVs that is generated prior to DC activation is by definition already loaded with self peptides. Therefore it would be non-productive or potentially even dangerous to recruit such a pool of preassembled pMHCII during DC maturation. In **chapter 4** we found that the vast majority of MHCII at the plasma membrane of mature DCs was newly synthesized during DC maturation and not derived from ILVs. MHCII could be recruited from a pre-synthesized pool in the endocytic pathway, but only when not yet sequestered in ILVs. Thus, maturing DCs generate new pMHCII complexes at the moment of pathogen encounter and degrade those pMHCII complexes that were already loaded with self-peptide and sorted to ILVs before DC maturation.

Figure (left). Processing of MHCII in immature and activated DCs
After synthesis in the endoplasmic reticulum, MHCII is transported to the endocytic tract with the aid of associated invariant chain (Ii) (1). Ii is degraded by endosomal proteases (2), thereby liberating MHCII for peptide loading (3). In immature DCs, pMHCII is ubiquitinated by MARCH I (4), driving pMHCII into the intra luminal vesicles (ILVs) of multi vesicular bodies (MVBs) (5). Some pMHCII may escape a first round of sorting at MVBs and consequently appear at the plasma membrane of immature DCs (6). These complexes can then be endocytosed (7) for a second opportunity to be sorted into ILV. In activated DCs (bottom panel), synthesis of MHCII is temporarily up-regulated and internalized pathogens are proteolytically processed into peptides, which can be loaded on newly synthesized MHCII (8). Ubiquitination of pMHCII ceases, as a consequence of which its transport to lysosomes is abrogated and reverted for stable expression at the plasma membrane (9).

There was controversy in the literature regarding the question whether peptide loading of MHCII is developmentally regulated through Ii processing. In **chapter 5** we report on an experimental artifact that could be the probable cause of these controversies. Like most cells, DCs are usually cultured in media that are supplemented with L-glutamine. This amino acid is, however, unstable and decomposes with time, generating NH_3. This weak base freely diffuses through membranes and is protonated in intracellular compartments with low pH. In cultured DCs, endosomal pH was sufficiently raised to dramatically affect the degradation of Ii by pH dependent endosomal proteases. We found that Ii degradation is slow in immature DCs and accelerated in activated DCs when the cells were cultured in L-glutamine containing media. However, when DCs were cultured in medium in which L-glutamine was substituted by a stable dipeptide as a glutamine source, degradation of Ii was already fast in immature DCs, in agreement with the idea that Ii processing, and thus MHCII peptide loading, are not developmentally regulated.

Experimental interference with Ii degradation by adding low amounts of NH_4Cl to culture media, blocked ubiquitination of MHCII and consequently also its sorting at MVBs, while Ii endocytosis motifs remained intact. This condition was used in **chapter 4** to experimentally accumulate MHCII at the endosomal delimiting membranes. Subsequent removal of the NH_4Cl resulted in a synchronous release of thus accumulated MHCII. This pool of MHCII was transferred to the plasma membrane in response to a maturation signal, confirming that MHCII could be recruited from the endosomal delimiting membrane but not from ILVs.

In **chapter 6** we combined the acquired knowledge on the effects of NH_4Cl on MHCII processing to experimentally enhance MHCII antigen presentation by immature DCs. The cohort of MHCII that was accumulated in the presence of NH_4Cl could be efficiently loaded with exogenously administered peptide after removal of the NH_4Cl block. MHCII that was released from the NH_4Cl block could be efficiently loaded by exogenously added peptides and such specific complexes were temporarily highly expressed at the immature DC plasma membrane. This procedure improved the potential of immature DCs to present pMHCII to T cells and could potentially be used for the development of novel therapeutic DC-based tolerization strategies.

The studies described in this thesis clarify several key regulatory events in the processing of MHCII and antigen presentation by DCs. Ubiquitination of MHCII is the key negative regulator that determines whether MHCII is expressed at the DC plasma membrane. It is not yet known where MHC class II ubiquitination occurs, at the endosomal delimiting membrane or at the plasma membrane or at both locations. In the context of efficiency of antigen presentation by immature DCs, this does impose an interesting question: Is MHCII in immature DCs, after peptide loading, committed to at least one transport cycle to the plasma membrane and back into the endocytic system? This question is particularly relevant for the tolerogenic function of immature DCs. Another remaining question is how ubiquitination of MHCII is shut off during DC maturation. Regulation of the expression of the responsible E3-ligases is part of the answer, but certainly also other regulatory mechanisms are in play. After DC activation, only a minority of MHCII molecules truly display pathogen derived peptides. This poses a conceptual problem that also has not yet been resolved: how does the immune system prevent the activation of self-reactive T cells by activated DCs that in addition to foreign peptides also display self-peptides in the context of MHCII? Further specificity may be achieved by autonomous control of phagosomes for MHC peptide loading or through selective directional transfer of MHCII from pathogen-loaded phagosomes to the immunological synapse. Clearly, further research is required to understand how presentation of pMHCII by DCs is regulated.


MHCII
cellen
cel
dendritische
endosoom
presentatie
antigeen
eiwitten
membraan
peptiden
membraaneiwitten
ubiquitinering
membraanblaasjes
plasmamembraan
afbraak
hoofdstuk
geactiveerde
celoppervlak
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immuunsysteem
luminale
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complex
expressie
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endocytose
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verhoging
vorming
ziekteverwekker
pMHCII
ubiquitine
belangrijk
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endosomen
beschreven
Wanneer
complexen
blaasjes
organellen
endosomale
uitgevoerd
moleculen
laten
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oppervlakte
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afweerreactie
lumen
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afhankelijk
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gereguleerd
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Via
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Nederlandse samenvatting voor de leek

Nederlandse samenvatting voor de leek

Dieren en de mens zijn opgebouwd uit cellen, waarvan er een groot aantal soorten bestaan, ieder met specifieke functies. De binnenkant van cellen is opgebouwd uit een kern, welke het erfelijk materiaal in de vorm van DNA bevat, en het cytoplasma. Het cytoplasma is samengesteld uit het cytosol en organellen. Het cytosol is de door het plasmamembraan begrensde waterige fase die is samengesteld uit opgeloste moleculen en ionen, en macromoleculaire complexen. Voorbeelden van organellen zijn het endoplasmatisch reticulum (ER), het Golgi apparaat, endosomen en lysosomen. Het plasmamembraan vormt het oppervlak van de cel dat de inhoud van de cel scheidt van de buitenwereld en wordt gevormd door een laag van vetten en eiwitten. Via het plasmamembraan communiceert de cel met zijn omgeving. Ook de verschillende celorganellen zijn omgeven door een membraan. De samenstelling van membranen wordt door behoeftes van de cel of door instructies van buitenaf continue aangepast. Hiervoor worden macromoleculen (eiwitten en vetten) tussen de organellen en van en naar het celoppervlak getransporteerd door middel van kleine membraan blaasjes. Cellen kunnen dus plasmamembraaneiwitten maar ook macromoleculen uit de omgeving insluiten in een membraanblaasje dat zich naar binnen toe afknopt van het plasmamembraan. Na dit internalisatie of “endocytose” proces wordt het membraan blaasje gedirigeerd naar het zogeheten endosoom. Dit organel is een soort centraal sorteringstation waarvandaan geëndocyteerde moleculen naar verschillende bestemmingen in de cel gestuurd kunnen worden, ook weer via membraanblaasjes. Om al deze membraantransporten efficiënt uit te voeren beschikt de cel over een “wegennet”, het cytoskelet genaamd. Via deze infrastructuur en ingebouwde codes in de te vervoeren eiwitten wordt de eindbestemming van transportblaasjes en de lokalisatie van membraaneiwitten binnen de cel bepaald. Naast intrinsieke codes, kunnen membraaneiwitten ook een “etiket” krijgen door het bevestigen van signalerings moleculen, zoals het eiwit ubiquitine. Eenmaal aan een eiwit gekoppeld kan er aan dit ubiquitine een tweede ubiquitine worden gekoppeld. Door herhaling van dit proces wordt er een ubiquitine streng gevormd aan een eiwit. Het aankoppelen

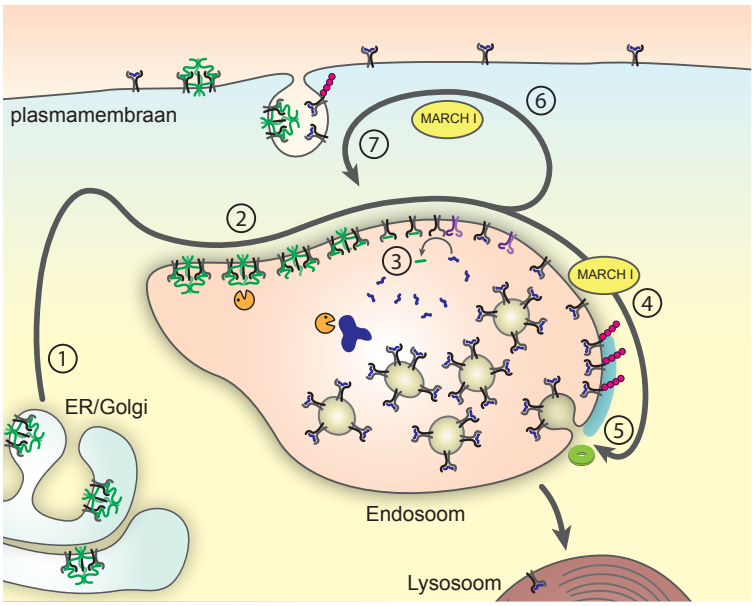
van ubiquitine wordt uitgevoerd door enzymen, ook wel ubiquitine-ligases genoemd. Geubiquitineerde membraaneiwitten kunnen herkend worden door sorteringscomplexen in het cytosol, die deze eiwitten kunnen sorteren van niet geubiquitineerde membraaneiwitten. Het hiervoor genoemde endosoom is erg dynamisch van aard en er wordt onderscheid gemaakt tussen een "vroeg" en een "laat" endosomaal stadium. Gedurende de overgang van het vroege naar het late endosoom worden geubiquitineerde membraaneiwitten gesorteerd door zogenoemde ESCRT complexen naar membraanblaasjes die zich afknoppen naar het lumen (binnenkant) van het endosoom. Op deze wijze wordt er een endosoom gevormd waarbij kleine membraanblaasjes zijn omgeven door een groter membraan, het zogenaamde "multivesicular body". Samensmelting van dit organel met het lysosoom, een organel dat beschouwd kan worden als het afvalvat van de cel, heeft tot ultiem gevolg dat de inhoud wordt afgebroken. Ubiquitinering van membraan eiwitten leidt dus uiteindelijk tot hun afbraak in het lysosoom.

Om zich te verweren tegen ziekteverwekkers zoals bacteriën, virussen en parasieten maar ook zieke lichaamscellen (zoals kankercellen), is het lichaam uitgerust met een zogeheten afweer- of immuunsysteem. Het vakgebied dat dit systeem bestudeert noemt men de immunologie. Het immuunsysteem is te verdelen in twee functionele onderdelen. Als eerste het aangeboren immuunsysteem dat bestaat uit cellen die natuurlijke barrières vormen, zoals aanwezig in de huid, darmen en longen, en uit immuuncellen die ziekteverwekkers direct kunnen elimineren. De werking van dit deel van het immuunsysteem is afhankelijk van evolutionair bepaalde en geconserveerde verschillen in moleculaire opbouw tussen het organisme en de ziekteverwekker. Het tweede onderdeel van het immuunsysteem wordt het adaptieve immuunsysteem genoemd en wordt gekenmerkt door een door de ziekteverwekker zelf geïnduceerde specifieke afweerreactie. Dit systeem wordt gevormd door een verscheidenheid aan cellen waaronder de dendritische cellen, die verantwoordelijk zijn voor het opstarten van een adaptieve immuunreactie. Onder normale condities patrouilleren deze cellen door het lichaam en "voelen" of er een bedreiging aanwezig is. Ook verzamelen zij daarbij continu materiaal, inclusief dat van een ziekteverwekker, uit hun omgeving via endocytose. Dit materiaal, waaronder eiwitten, belandt na endocytose in

endosomen alwaar de eiwitten worden afgebroken in kleinere fragmenten (zie figuur). Deze fragmenten, ook wel peptiden of antigenen genoemd, kunnen in het endosoom associëren met een membraan eiwit complex dat het major histocompatibility complex klasse II (MHCII) genoemd word. MHCII wordt geproduceerd in het ER en associeert daar met een ander eiwit, de invariante keten (Ii), waardoor er een MHCII-Ii complex gevormd wordt. De Ii begeleidt MHCII vanuit het ER, via het Golgi en transportblaasjes naar het endosoom. Een tweede functie van de Ii is dat het de bindingsplaats voor peptiden op MHCII bezet. Zo kan er tijdens het transport van MHCII geen ongewenste binding van peptiden plaats vinden. Na aankomst bij het endosoom wordt de Ii verwijderd en kan MHCII de daar geproduceerde peptiden binden, in een proces dat wordt gefaciliteerd door het DM eiwit (zie figuur). Afbraak van de Ii en het genereren van peptiden wordt uitgevoerd door endosomale enzymen. De activiteit van deze enzymen is veelal afhankelijk van de lage zuurgraad (pH) die in het endosoom heerst. Op deze manier worden er in het endosoom heel veel verschillende peptide-MHCII complexen (pMHCII) gevormd welke vervolgens naar het plasmamembraan kunnen worden getransporteerd. Daar kunnen deze complexen worden herkend door specifieke T cellen (zie figuur). Het op deze manier uitstallen van peptiden op het celoppervlak wordt antigeen presentatie

Figuur (rechts). Model voor endosomale MHCII processing in niet geactiveerde en geactiveerde dendritische cellen
Nadat MHCII is gesynthetiseerd in het ER/Golgi systeem, wordt het getransporteerd naar het endosoom met behulp van de geassocieerde invariante keten (Ii) (1). De Ii wordt daar dan afgebroken door endosomale enzymen (proteasen) (2) waardoor het mogelijk wordt voor MHCII om een peptide te binden, hetgeen verder wordt gefaciliteerd door het DM eiwit (3). In niet geactiveerde dendritische cellen wordt pMHCII geubiquitineerd door MARCH I (4) waarna geubiquitineerd pMHCII gesorteerd wordt door het ESCRT complex naar de luminale membraanblaasjes van het endosoom (5). Sommige pMHCII ontsnappen aan deze eerste sorterings ronde en worden naar het plasmamembraan getransporteerd (6). Deze pMHCII complexen kunnen worden geëndocyteerd (7) en na aankomst in het endosoom alsnog naar luminale membraanblaasjes gesorteerd worden. In geactiveerde dendritische cellen (onderste paneel), is de synthese van MHCII tijdelijk verhoogd en worden opgenomen ziekteverwekkers (pathogenen) in het endosoom afgebroken. Peptiden van het afgebroken pathogeen kunnen binden aan nieuw geproduceerd MHCII (8). Door activering van de dendritische cel stopt de ubiquitinering van pMHCII waardoor afbraak in het lysosoom wordt voorkomen en alle nieuw gevormde pMHCII stabiel op het celoppervlak terecht komt (9). Hierdoor kan de dendritische cel een "snap-shot" maken van antigenen welke gevonden zijn tijdens de ontmoeting met een ziekteverwekker, en deze dan aan T cellen tonen.

niet geactiveerde dendritische cel



MHCII-Ii

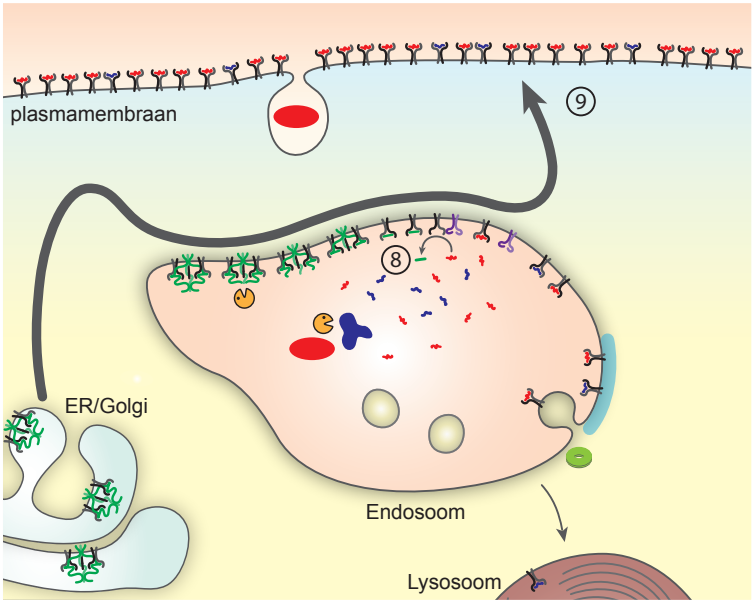
MHCII-eigen peptide

MHCII-pathogeen peptide

geubiquitineerd MHCII

DM

geactiveerde dendritische cel



proteasen

eigen-eiwit

pathogeen

VPS4

ESCRT

genoemd. Dendritische cellen worden dan ook wel professionele antigeen presenterende cellen genoemd. Wanneer een bedreiging wordt geconstateerd, worden locale dendritische cellen geactiveerd en veranderen deze tijdens een proces dat ook wel "maturatie" wordt genoemd. In de geactiveerde status beweegt de dendritische cel zich naar lymfe klieren of de milt en geeft daar alarmsignalen af. In deze organen bevinden zich ook T cellen, waar bijna een oneindig aantal varianten van zijn, ieder met een eigen T cel receptor die maar op een enkele soort peptide past. Wanneer een T cel een pMHCII complex op een dendritische cel herkent wordt er een afweerreactie gestart, mits ook andere benodigde alarmsignalen aanwezig zijn. Een bijzonder aspect van de dendritische cel is dat zij na detectie van een indringer in staat is om de antigeen presentatie sterk te verhogen. Een belangrijk punt is dat dendritische cellen ook lichaamseigen peptiden presenteren maar bij een lage presentatie en in de afwezigheid van additionele alarmsignalen zullen herkennende T cellen de gepresenteerde "eigen" peptiden tolereren. Wanneer de balans toch verstoord is en een afweerreactie gestart wordt tegen lichaamseigen eiwitten kan dit resulteren in een auto-immuun ziekte.

De rode draad door dit proefschrift draait om de vraag hoe dendritische cellen voor, tijdens en na activering in staat zijn om de antigeen presentatie via MHCII te reguleren (zie figuur). Na een overzicht gegeven te hebben in **hoofdstuk 1** van de bestaande kennis over de functie en regulatie van MHCII wordt in **hoofdstuk 2** beschreven hoe de oppervlakte expressie van MHCII wordt gereguleerd in de dendritische cel. MHCII heeft een geconserveerd en uniek lysine residu welke geubiquitineerd kan worden zodra de Ii verwijderd is. Het belangrijkste ubiquitine-ligase dat dit bewerkstelligt in het geval van MHCII is MARCH I, welke elders in de vakliteratuur is beschreven. Deze ubiquitinering zorgt voor een efficiënte sortering van MHCII naar de membraanblaasjes in het lumen van het endosoom, hetgeen nodig is om MHCII uiteindelijk af te breken in het lysosoom. Deze route is belangrijk om de oppervlakte expressie van MHCII relatief laag te houden in ongestimuleerde dendritische cellen. Behalve voor afbraak lijkt ubiquitinering van MHCII ook haar internalisatie te bevorderen, hoewel het nog onduidelijk is hoe groot het belang hiervan is. Activering van de dendritische cel leidt tot een drastische afname van MHCII ubiquitinering en daarmee tot een sterke verhoging van MHCII aan het celoppervlak. Wanneer

MHCII werd gemuteerd zodat het niet kon worden geubiquitineerd was de expressie op het celoppervlak al hoog op niet geactiveerde dendritische cellen. Deze resultaten geven aan dat ubiquitinering van MHCII belangrijk is in de regulering van antigeen presentatie.

Om membraaneiwitten af te breken moeten zij eerst worden geïncorporeerd in de luminale membraanblaasjes van endosomen. Ubiquitinering van membraaneiwitten is hierbij een belangrijk signaal voor herkenning door het "ESCRT" sorterings complex. ESCRT bestaat uit een aantal componenten die samen membraan eiwitten selecteren en begeleiden naar het endosomale lumen. Tevens vormen zij een drijvende kracht voor de vorming van de luminale membraanblaasjes waarnaar de membraaneiwitten gesorteerd worden. De laatste schakel in dit proces wordt gekatalyseerd door het VPS4 eiwit en is onmisbaar voor het afknoppen van de luminale membraanblaasjes en dus ook de afbraak van membraan eiwitten. In **hoofdstuk 3** is onderzocht wat het effect is op de distributie van MHCII wanneer de vorming van luminale membraan blaasjes wordt geremd door de werking van VPS4 te blokkeren. Hiervoor werd een dominant-negatief VPS4 mutant eiwit in de dendritische cellen gebracht. In deze setting bleek de oppervlakte expressie van MHCII verhoogd te zijn, evenals de totale hoeveelheid MHCII. Opvallend genoeg was er geen effect op de formatie van pMHCII, wat aangeeft dat de vorming van luminale membraan blaasjes wel belangrijk is voor MHCII sortering naar lysosomen maar niet voor de peptide belading van MHCII.

Wanneer dendritische cellen niet gestimuleerd worden, concentreert MHCII zich op de luminale membraan blaasjes in het endosoom. In een geactiveerde dendritische cel bevindt het merendeel van MHCII zich juist op het plasmamembraan. Lange tijd was het niet duidelijk wat de bron was van het MHCII dat uiteindelijk de antigeen presentatie verzorgt. De resultaten in **hoofdstuk 4** laten zien dat antigeen presentatie door een geactiveerde dendritische cel voornamelijk door nieuw geproduceerd pMHCII wordt uitgevoerd. Dit werk geeft ook aan dat pMHCII dat al gevormd is en zich reeds bevindt op de luminale membraan blaasjes vóór de ontmoeting met een ziekteverwekker, toch afgebroken wordt en niet participeert in de antigeen presentatie. Een dergelijk mechanisme is gewenst om te voorkomen dat de MHCII moleculen die al voor activering van de dendritische cel zijn beladen met

van "eigen" eiwitten afkomstige peptiden, het plasmamembraan bereiken, en daarmee potentiëel een auto-immuun reactie zouden kunnen opwekken.
In **hoofdstuk 5** laten we zien dat de afbraak van Ii niet wordt versneld in een geactiveerde dendritische cel, hoewel dit in andere studies wel gesuggereerd werd. Verder hebben we aangetoond dat het gebruik van glutamine in celkweek medium leidt tot pH verhoging van het endosomale milieu. Dit blijkt veroorzaakt te worden door de ophoping van ammoniak (NH_3) in het medium, één van de producten die ontstaan na het uiteenvallen van het relatief onstabiele glutamine. Dit artefact heeft een sterk effect op de pH afhankelijke afbraak van de Ii in endosomen en kan ook de controverses over Ii afbraak in de literatuur verklaren. Als bevestiging van bevindingen in hoofdstuk 2 is ook gebleken dat ubiquitinering van MHCII pas plaats vindt na de pH afhankelijke afbraak van de MHCII-geassocieerde Ii.

Voortbordurend op hoofdstuk 4 en 5 wordt in **hoofdstuk 6** een experimentele procedure beschreven waarmee antigeen presentatie door niet geactiveerde dendritische cellen verhoogd kan worden. We hebben hiervoor eerst MHCII-Ii complexen laten accumuleren binnenin de cel door de afbraak van Ii te remmen. Door de Ii afbraak daarna weer toe te laten kon dit cohort van MHCII worden "bevrijd". Deze procedure bleek een tijdelijke verhoging van MHCII op het celoppervlak en daarmee verbetering van antigeen presentatie naar T cellen te bewerkstelligen. De beschreven methode zou in de toekomst mogelijk het ontwerp van op dendritische cellen gebaseerde vaccin strategieën kunnen ondersteunen.

Met de in dit proefschrift beschreven resultaten is meer inzicht verkregen in de fundamentele moleculaire processen die het transport en peptide belading van MHCII reguleren (zie figuur), en de betekenis daarvan voor het opwekken van specifieke immuunreacties. De ubiquitinering van MHCII is een cruciaal proces in de regulatie van antigeen presentatie. Hoe de ubiquitinering van MHCII zelf wordt gereguleerd tijdens activering van de dendritische cel is nog niet opgehelderd. Manipulatie van Ii afbraak, zoals besproken in hoofdstuk 6, of van MHCII ubiquitinering, zouden goede opties zijn voor nieuwe vaccinatie strategieën. MHCII processing in dendritische cellen is fundamenteel voor de initiatie van adaptieve immuunreacties. Kennis van de moleculaire en cellulaire mechanismen van MHCII processing is daarom cruciaal voor de ontwikkeling

van nieuwe vaccinatie strategieën en therapieën tegen auto-immuun ziekten.

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Opleiding
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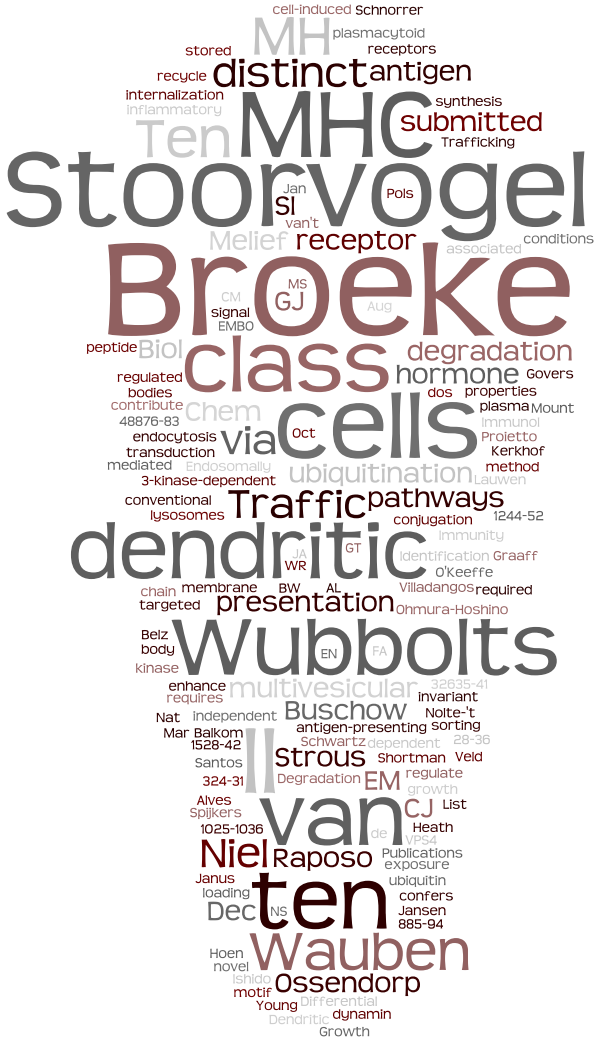
Willem
uitdaging
systeem
extracellulaire
Tijdens
Enrico
bestudeerd
Hierbij
gaan
studie
Biochemie
Wetenschappen
bachelordiploma
Roland
samenhangende
Wauben
verdieping
genoemde
binnen
onderzoek
MHC

Curriculum Vitae

Curriculum Vitae

Toine ten Broeke werd geboren op 7 april 1975 te Duiven. Hij behaalde zijn VWO diploma in de zomer van 1994 aan het Nederrijn College te Arnhem. In september van hetzelfde jaar begon hij aan de Hogere Laboratorium Opleiding (HLO) aan de Hogeschool van Arnhem en Nijmegen, met Biochemie als afstudeerrichting. Gedurende deze studie werden twee stages doorlopen aan de vakgroep Celbiologie in het Universitair Medisch Centrum Utrecht. Onder de begeleiding van Drs. Roland Govers en Prof. Dr. Ger Strous werd de ubiquitinering en internalisatie van de groeihormoonreceptor bestudeerd. In de zomer van 1998 werd het bachelordiploma behaald en aansluitend begon hij in de hiervoor genoemde onderzoeksgroep als research analist. Eind 2000 maakte hij binnen deze vakgroep de stap om onder leiding van Dr. Willem Stoorvogel als senior research analist verder te gaan en zich te verdiepen in het intracellulaire transport van MHC klasse II. Wegens de tot hoogleraar benoemde Willem Stoorvogel en de daarmee samenhangende verhuizing kon hij in 2004 en 2005 op detacheringbasis dit werk voortzetten bij de afdeling Biochemie & Celbiologie, faculteit Diergeneeskunde, Universiteit Utrecht, onder de regie van Prof. Dr. Willem Stoorvogel. In de eerste helft van 2006 werd de functie als senior research analist aan de vakgroep Celbiologie in het Universitair Medisch Centrum Utrecht vervolgd onder de leiding van Dr. Peter van der Sluijs. Tijdens deze periode werd de functie van bij endosomaal transport betrokken eiwitten bestudeerd. Op zoek naar meer verdieping en uitdaging besloot hij om in september 2006 te starten met het in dit proefschrift besproken promotieonderzoek. Dit werd gedaan onder de leiding van Prof. Dr. Willem Stoorvogel en Dr. Richard Wubbolts bij de afdeling Biochemie & Celbiologie, faculteit Diergeneeskunde, Universiteit Utrecht.

Sinds maart 2012 werkt Toine als post-doc in een samenwerkingsproject tussen Prof. Dr. Marca Wauben van de afdeling Biochemie & Celbiologie, faculteit Diergeneeskunde en Dr. Enrico Mastrobattista, faculteit Bètawetenschappen, afdeling Farmaceutische Wetenschappen, beide van de Universiteit Utrecht. Hierbij wordt onderzoek gedaan naar (extracellulaire) membraanblaasjes en mogelijkheden om deze te gebruiken als gericht transport en aflever systeem voor therapeutica.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Growth

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would
voorzien
Familie
Paris
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Marnix
werpen
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Jan-Willem
werkte
af
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Daan
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Dankwoord

Dankwoord

Het is zover!
Mijn proefschrift is af.
Het is tijd om eens een terugblik te werpen. Promoveren doe je niet alleen en daarom wil ik alle mensen bedanken die hier op wat voor manier dan ook aan hebben bijgedragen.

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Σ 'αγαπώ

Toine.