Regulation of MHC class II surface expression in dendritic cells by ubiquitination

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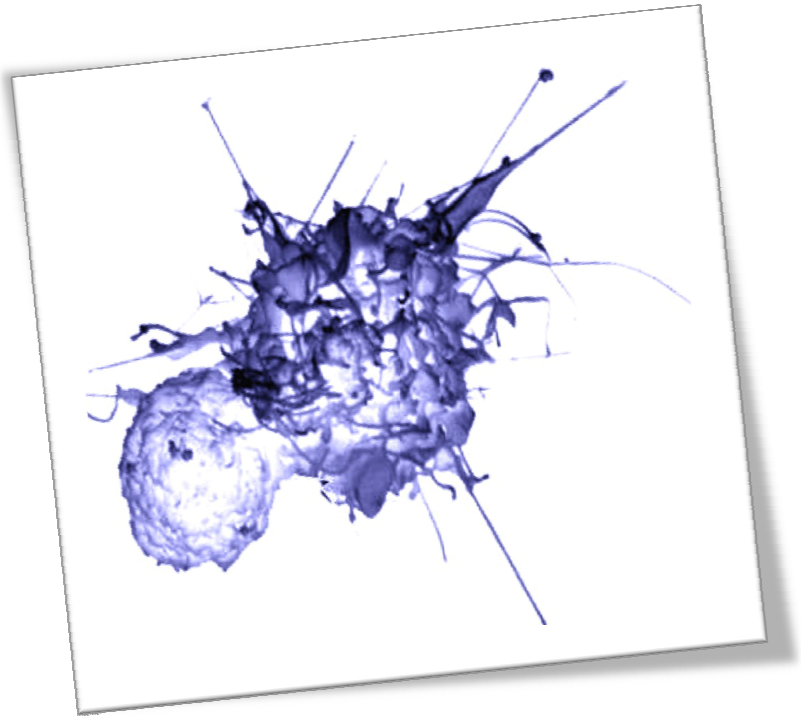

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Cover picture: A mature monocyte-derived dendritic cell interacts with the T cell receptor of a CD4 T cell via MHC class II molecules. Usually, this results in the activation of the CD4 T cell. Thus, dendritic cells form the connection between invading pathogens and the adaptive immune system (http://www.lhir-lirh.ulaval.ca).

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Abstract

Dendritic cells (DC) are antigen presenting cells specialized in antigen uptake, processing and presentation. Maturation of DC is an essential process in order to activate T cells in the lymph nodes and is characterized by upregulation of MHCII on the cell surface. Antigen presentation via MHCII is of great importance in the immune system and is a central process in adaptive immunity. In immature DC most of the newly formed peptide-MHCII (pMHCII) complexes are rapidly ubiquitinated, either directly or after expression on the plasma membrane, driving the active sorting of pMHCII to intraluminal vesicles at multivesicular bodies. MHCII synthesis is temporarily enhanced in maturing DC and ubiquitination of MHCII is simultaneously prevented, resulting in a high expression of stable MHCII loaded with antigenic peptides. The process and regulation of MHCII ubiquitination is, however, incompletely understood. Recent insights have been provided by discovery of the role of MARCH ligases and ITAM signaling in regulating ubiquitination. By understanding MHCII regulation it might be possible to modulate antigen presentation, preferably in specific subsets of DC. Stimulating the presentation of tumor-antigens on MHCII could improve cancer treatment in patients and might result in 'tumor memory'. Furthermore, in all other diseases and infections where the adaptive immune system is involved, understanding the regulation of MHCII expression and antigen presentation will be of great value.

Introduction

Ever since it was discovered that T cell immunity relies on peptides generated from proteins, instead of recognizing the tertiary structure of proteins as in B cell immunity, antigen processing and presentation have been extensively studied. Consequently, we now understand the differences between antigen recognition in B and T cells and this knowledge is valuable information for designing vaccines and other immune-based therapies. Central to the activation of T cells is recognizing antigenic peptides present in the peptide-binding cleft of major histocompatibility complex (MHC) class I or class II molecules[1]. The function of MHC class I (MHCI) molecules is to display fragments of intracellular proteins (peptides) to CD8 cytotoxic T cells[2]. In case of infection with a virus, intracellular bacteria, or transformation of the cell, foreign peptides are presented on MHCI and the immune system will respond by killing these cells. Peptides generated for binding to MCHI are delivered to the endoplasmic reticulum (ER) via TAP (transporter associated with antigen processing)[3]. Loading of MHCI with peptides occurs in the ER and peptide-loaded MHCI is then expressed at the cell surface of all nucleated cells and platelets. Expression of MHCII is found on professional antigen-presenting cells (APCs), including B cells, macrophages and dendritic cells (DC). DC actively sample the environment and are specialized in antigen uptake, processing and presentation[2]. The function of MHC class II (MHCII) molecules is to present processed antigens to CD4 helper T cells. Antigen-specific CD4 T cells become activated through interaction of peptide-MHCII (pMHCII) and the T cell receptor (TCR) (Bousso, 2008). CD4 T cells then activate specific B cells, resulting in B cell differentiation into antibody producing plasma B cells. If pMHCII complexes are not properly formed in APCs, both in mice and in patients, this results in an inefficient antibody response against infections[4]. Besides that, antigen presentation in the context of MHCII controls the (indirect) activation of cytotoxic T cells, autoimmune responses, and other immune responses against pathogens or the environment.

DC play a crucial role in activating naïve T cells and are able to direct the response of the adaptive immune system. But how are cytotoxic T cells activated if intracellular microorganisms do not infect DC or other APCs? This is possible via a process called cross-presentation. DC take up and digest infected cells (or fragments from these cells), generate peptides and subsequently present these peptides on MHCI molecules at the plasma membrane to CD8 T cells[5]. In this way, the cytotoxic T cell response is activated and other infected cells can be eliminated. This is an atypical process and the mechanism through which peptides from endocytosed pathogens can access MHCI molecules remains to be elucidated.

MHCII presentation is a central process during the induction of adaptive immunity, thus understanding and studying the cellular mechanism behind is of crucial importance. Not only to identify factors that cause a variety of diseases linked to MHCII presentation, but also to discover means of regulating and controlling antigen presentation. Antibodies, other important proteins of the immune system, are used to identify and neutralize pathogens and foreign cells. Furthermore, monoclonal antibodies (mAb) are used in cancer therapy to specifically bind to tumor cells[6]. In this way, the immune system in stimulated to attack those cells. Specifically stimulating the presentation of tumor-antigens on MHCII could improve mAb cancer therapy in patients. On the other hand, finding agents that block the presentation of certain autoantigen-derived peptides might lead to new treatments for autoimmune diseases and allergic responses.

To be able to modulate antigen presentation via MHCII it is important to understand how MHCII expression at the cell surface is regulated. If MHCII is not expressed at the cell surface (as in immature DC), antigenic peptides cannot be presented to T cells and the immune system will not be activated. It has been shown that mono-ubiquitination is involved in down-regulation of receptors through targeting these receptors to the endo-lysosomal pathway[7]. Ubiquitination is the process where one or multiple ubiquitin proteins are added to a substrate. Studies from the past few years have provided evidence that ubiquitination indeed is involved in regulating MHCII expression on the cell surface of DC[8]. However, the precise mechanism of this regulation is not fully understood. And moreover, which factors direct MHCII ubiquitination remains incompletely understood either. Combining these questions with the promising future applications of regulating MHCII presentation, has led to the following main question of this thesis:

How is MHC class II surface expression regulated in dendritic cells by ubiquitination?

In order to answer this question, we will first explore the maturation process that DC undergo after antigen encounter and describe the differences between DC subsets. Next, it is necessary to comprehend the synthesis, surface expression and transport of MHCII. After that, the role of ubiquitination in regulating MHCII expression will be discussed. Last, a model is presented that combines MHCII synthesis, transport, ubiquitination, surface expression, and the differences between immature and mature DC. In the appendices the results of the experiments performed as part of this thesis can be found.

Chapter 1. Maturation of dendritic cells

Dendritic cells (DC) are frequently described according to the 'Langerhans cell paradigm'[9]. This paradigm states that immature DC are present in peripheral tissues, and upon capture of activating antigen will migrate and mature towards secondary lymphoid organs, e.g. the lymph nodes. Immature DC sample their environment efficiently through constitutive macropinocytosis and phagocytosis and have low levels of MHC class II (MHCII) molecules and other T-cell stimulatory molecules, like CD40 and CD86, on the cell surface[10]. This makes these immature DC specialized in the uptake of antigens from the environment. Immature DC also express a variety of receptors for pathogen-associated molecular patterns (PAMPs) and other inflammatory molecules on the cell surface. Examples of these receptors are Toll-like receptors (TLRs), C-type lectin receptors, and mannose receptors[11].

Stimulating these receptors will generate an intracellular signal in the immature DC, triggering DC migration and maturation. Maturation of DC is characterized by expressing high levels of MHCII and CD86 molecules on the cell surface[12]. Mature DC are specialized in presenting peptides from internalized antigens on MHCII to CD4 T cells in the lymph nodes[9]. In this way, DC form the link between the entering sites of pathogens, e.g. the gut and lungs, and sites where T cells are located, e.g. lymph nodes and spleen.

Subsets of DC

Since the first purification of DC from lymphoid organs, other subsets of DC have been identified and show considerable heterogeneity[13]. Not every subset follows the previously described cycle of immature DC in the periphery migrating to the lymph nodes while activated and maturing once in the lymph nodes. Two main classes of DC can be distinguished: plasmacytoid and myeloid DC. The first class, plasmacytoid DC, are DC circulating through blood and lymph, thereby passing the lymphoid tissues[12]. These DC only adopt the classical DC morphology after activation and are able to produce interferon-1 (IFN-1). This explains the former name of these cells: Type-1 Interferon Producing Cells or IPCs[14]. The function of plasmacytoid DC in the immune system is unclear and that is why the name IPC is still used sometimes. The second class are the myeloid DC, present in the thymus, the spleen, the lymph nodes, and in peripheral tissues. In this thesis, whenever the term 'DC' is used, the myeloid DC are meant. Myeloid DC can be further subdivided into five subtypes, which are listed with their characteristics in Table 1[12].

Lymphoid-organ-resident DC

As can be seen in Table 1, myeloid DC can be further divided in two subsets, each consisting of two or three subtypes. The lymphoid-organ-resident DC subsets, as the name indicates, are found in the spleen, thymus and lymph nodes[15]. The lymphoid-organ-resident DC develop from precursors in the bone marrow within lymphoid organs, therefore not seeing the periphery before that. These DC remain an immature phenotype during their life if not triggered by an infection. However, if these cells are cultured *in vitro* they spontaneously maturate, thus start to show high MHCII and CD86 expression on the cell surface[10]. If pathogens or stimuli associated with pathogens enter the lymph nodes, maturation is also triggered. Three subtypes of lymphoid-organ-resident DC can be identified, according to their CD4 and CD8 expression on the cell surface: CD4+ DC, CD8+ DC and CD4-/CD8- DC, the latter is also termed double negative DC[12].

Table 1. Dendritic cell subtypes in the mouse

Features	Lymphoid-organ-resident DC subsets			Migratory DC subsets	
	CD4+ DC	CD8+ DC	CD4-/CD8- DC	Interstitial DC	Langerhans cells
Location of DC					
Spleen	Yes	Yes	Yes	No	No
Subcutaneous lymph nodes	Yes	Yes	Yes	Yes	Yes
Visceral lymph nodes	Yes	Yes	Yes	Yes	No
Thymus	Yes	Yes	Yes	No	No
Surface markers					
CD11c	+++	+++	+++	+++	+++
CD4	+	-	-	-	-
CD8	-	++	-	-	-/+
CD11b	++	-	++	++	++
CD24	+	++	+	Unknown	Unknown
Features in					
Maturation	Immature	Immature	Immature	Mature	Mature
Co-stimulation	+	+	+	++	++
Antigen processing and presentation	+++	+++	+++	+/-	+/-
***In vitro* equivalent**					
Bone marrow precursors plus:	FLT3L	FLT3L	FLT3L	GM-CSF, TNFα, TGFβ	GM-CSF, TNFα, TGFβ

Table 1. Dendritic cell subtypes in the mouse. Characteristics of different subtypes of DC, as studied in the mouse. Abbreviations: FLT3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte/macrophage colony-stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor. Simplified from Villadangos and Schnorrer, 2007[12].

Migratory DC

The other subset consists of the migratory DC. These DC develop from precursors in the peripheral tissues and then migrate to local lymph nodes. Approximately half of all lymph node DC are migratory, the other half are lymphoid-organ-resident DC[15]. The spleen and the thymus contain almost no migratory DC, because these organs are not provided with afferent lymph. Migratory DC follow the Langerhans cell paradigm, as mentioned before. However, it has been shown that migratory DC under special pathogen free (SPF) or germ free (GF) conditions already have the mature phenotype[16]. Maturation was measured via high surface expression levels of MHCII, CD40 and CD86, which are the classical markers for maturation[10]. It was thought that activation and maturation was triggered by activation of pathogen-recognition receptors, such as TLRs. TLRs need adaptor proteins MyD88 and TRIF for their intracellular signaling. To test the influence of these adaptor proteins in the maturation process, mice knocked out for these proteins were used. The migratory DC from the lymph nodes of single or double knockout mice also had the mature phenotype. This led to the

conclusion that migratory DC probably migrate and mature constitutively and that this happens independent of pathogen recognition and TLR signaling[16].

In vitro generated DC

To study DC biology, different methods to differentiate monocytes into DC are described. Monocyte-derived DC (MoDC) also play a role *in vivo* during inflammation, for instance when they accumulate in the skin of leprosy patients[17]. Most often bone-marrow (or spleen) precursor cells are cultured in a medium that contains granulocyte/macrophage colony-stimulating factor (GM-CSF). DC generated in this way show great resemblance to MoDC. Monocyte-derived DC are the most commonly used DC type in DC experiments[13]. The disadvantage of using these DC is that it remains unclear if these DC resemble any type of the original DC found in lymph nodes. They certainly show little resemblance to the lymphoid-organ-resident DC. These in vitro matured DC can be generated by adding GMS-like tyrosine kinase 3 ligand (FLT3L) to bone-marrow precursor cultures. DC created with FLT3L have no CD4 or CD8 expression, thus only double negative lymphoid-organ resident DC are generated[18]. Therefore, whenever experiments are done with MoDC created either by adding GM-CSF or FLT3L, the interpretation of results should be with caution and might not be extrapolatable to all the DC subtypes.

Triggers of maturation

DC maturation was first observed in isolated Langerhans cells. When these cells were cultured *in vitro* they lost the ability to present antigens yet gained the ability to stimulate T cells[19]. Some years later it was shown that upon maturation peptides of the antigen are rapidly loaded onto MHCII molecules. The rate of MHCII synthesis increases, as well as the half-life of MHCII, resulting in accumulation of long lasting stable MHCII complexes on the cell surface[20]. These long lasting MHCII complexes are needed to provide a good interaction for T cell priming.

But what signals trigger this maturation? In general, maturation is induced by microbes and viruses (danger signals) but it can also be mimicked by some microbial molecules, like lipopolysaccharide (LPS) or CpG-oligodeoxynucloeotides (CpG). As mentioned before, immature DCs can recognize PAMPs with their TLRs. For example, TLR-4 recognizes LPS and TLR-2 recognizes lipotechoic acid. Other TLRs are intracellular and recognize nuclear acids, with double stranded RNA (dsRNA) being the most potent trigger[11]. All TLRs are coupled to MyD88 and binding of a ligand can activate NF-κB, which then translocates to the nucleus and functions as a transcription factor[22]. Here, expression of thousands of genes are altered as part of the maturation process[23]. Cytokines

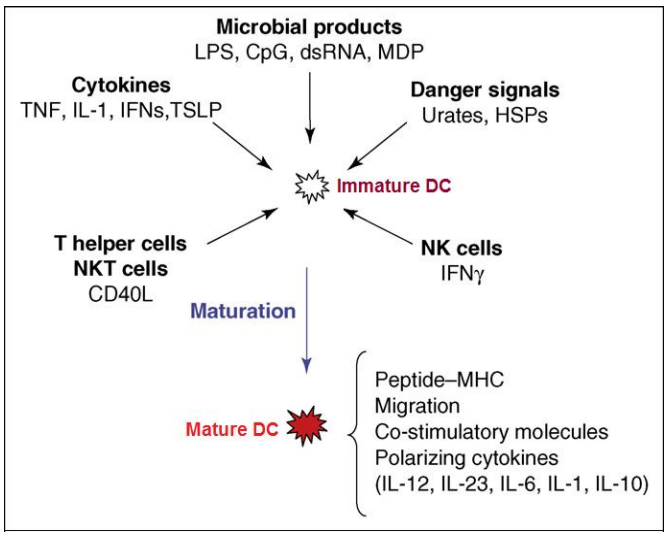

Figure 1. Stimuli that can trigger DC maturation. After an immature DC receives one or more of the stimuli, the maturation process will be activated. This results in an upregulation of peptide-MHC complexes, migration to lymph nodes and providing the ability to stimulate T cells. Adjusted from Magacno *et al.*, 2007[11].

produced by the DC itself (auto stimulation) or by other cells surrounding the DC are recognized by cytokine receptors. Three other classes of receptors are: (1) C-type lectin receptors, which sense bacterial and fungal carbohydrate moieties, (2) RIG-1 like receptors, which sense viral nucleic acids, and (3) Nod-like receptors, which are cytosolic sensors for bacterial products and danger signals (like heat shock proteins). Direct stimulation of DC can also be accomplished if T helper cells bind the MHCII-peptide complex, via the CD40-CD40L interaction. Figure 1 shows an overview of triggers for DC maturation[11]. Not all DC will be triggered by the same stimuli, because of the different locations of DC and because different subtypes can have different types of receptors upregulated on the cell surface. Many of these natural stimuli can also be used *in vitro* to trigger maturation.

Studies have shown that *in vitro* culturing can trigger maturation[21]. Freshly isolated DC from the spleen were cultured *in vitro* and their phenotype changed instantly: the average fluorescence level of CD86 expression increased a 100 times in 18 hours after culturing and the MHCII expression increased 4.4 times (Figure 2A)[10]. The differences after *in vitro* culturing resembled the changes that happen in DC derived from bone marrow (BMDC) after stimulating the cells with lipopolysaccharide (LPS). This indicates that lymphoid-organ DC can mature after culture *in vitro*. Furthermore, stimulation with LPS is also acknowledged as a means of inducing maturation. The changes that happen after LPS stimulation are visualized with immunofluorescence confocal microscopy (ICM) with MHCII and Lamp 1 labeling (Figure 2B). Lamp 1 is used to label the late endosomal or lysosomal compartments, where most of the MHCII molecules are stored in immature DC. Upon maturation, MHCII relocates to the plasma membrane and Lamp 1 compartments cluster near the nucleus. With these markers differences between epidermal DC from a lymph node and DC from the spleen can be shown after culturing (Figure 2C). Remarkably, no changes happen to lymph node DC after culturing

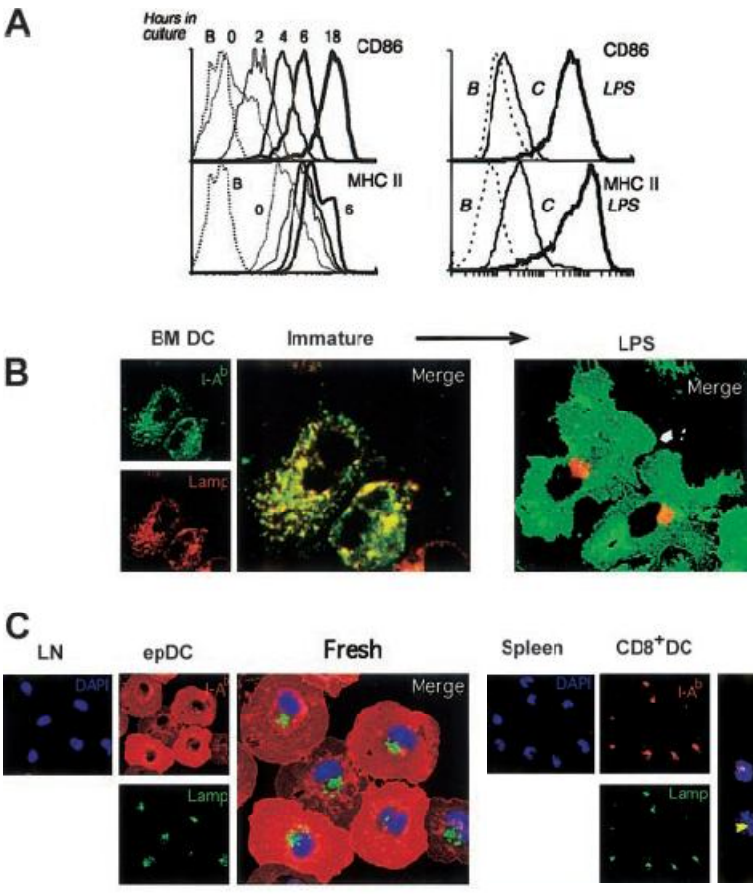

Figure 1. *In vitro* culturing and LPS stimulation can trigger maturation in splenic DC. (A) Left panel: Freshly isolated DC from the spleen were cultured for different times and FACS analysis was performed for CD86 and MHCII expression. Dashed lines are unlabeled cells. Right panel: Bone marrow derived DC (BMDC) incubated with or without LPS. (B) ICM analysis of BMDC cultured with or without LPS. Antibodies against MHCII (I-A^b, green) and Lamp 1 (red) were used to stain the cells. (C) Cells from the lymph node or spleen were cultured and stained for MHCII in red, Lamp 1 in green, and nucleus in blue (with DAPI). Adapted from Wilson *et al.* 2003[10].

and their phenotype resembles that of the mature state. However, DC derived from spleen cultures display dramatic redistribution of MHCII and morphological changes are visible[10]. Thus, merely culturing the DC from the spleen can trigger maturation. The same accounts for non-epidermal DC present in the lymph nodes (i.e. the lymphoid-resident DC). Epidermal DC from the lymph node already have acquired the mature phenotype. The markers presented by Wilson *et al*. and stimulation of LPS are powerful tools to visualize the maturation process of different subsets of DC.

Maturation of DC is an essential process in order to activate T cells in the lymph nodes, which is an important step in pathogen-specific immunity. The different subsets of DC show their importance in a range of locations and actions. However, the exact function of each of these subsets remains to be investigated, as well as the differences in antigen presentation and MHCII regulation.

Chapter 2. MHC class II synthesis and intracellular transport

In order to understand how MHCII expression is regulated, which will be discussed in the next chapter, we first need to comprehend the synthesis and surface expression of MHCII. The endocytic pathway plays an important role in MHCII synthesis, as will become clear in this chapter.

MHC class II molecules consist of an α- and β-membrane spanning protein, which are both highly polymorphic. Both subunits contain an immunoglobulin (Ig)-like domain and one transmembrane domain (Figure 3A)[24]. When these subunits are associated, a cleft is formed at the top where peptides can bind (Figure 3B)[25]. The peptide will alter the local structure of MHCII and the right T cell receptor is then able to bind this specific peptide-MHCII complex.

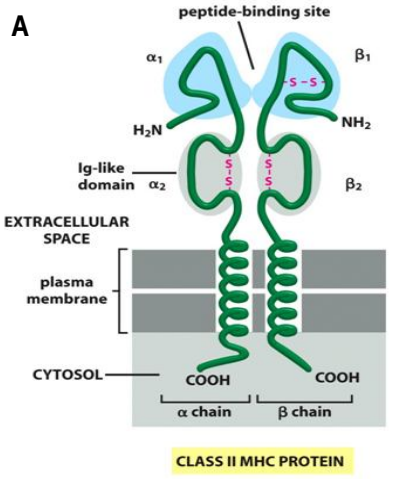

Figure 3. Schematic and molecular structure of MHCII molecules. (A) Schematic representation of MHCII, consisting of an α- and β-chain, both spanning the membrane one and containing an Ig-like domain. The αβ dimer forms a peptide-binding cleft. (B) Top view of the molecular surface of MHCII with a peptide bound in it. Aminoacids of peptide are indicated with one-letter code. Combined figure from Alberts *et al.*, 2007 and Stern *et al.*, 1994[24,25].

During MHCII synthesis in the endoplasmic reticulum (ER) the α- and β-chains are produced separately and then complexed with the invariant chain (Ii). Ii is a specialized polypeptide and present as a trimer in the ER. Therefore, Ii can bind three αβ complexes, which leads to the formation of nonameric $(\alpha\beta Ii)_3$ complexes[8]. Ii has a dual function: (1) occupying the peptide-binding cleft of MHCII, and (2) facilitating the transport of MHCII from the ER to endosomes[26,27]. Blocking the peptide-binding cleft by Ii prevents binding of cellular peptides (self-peptides) or peptides present in the endogenous pathway (such as peptides normally loaded onto MHC class I). The invariant chain is necessary for the export of MHCII from the ER to the Golgi, which is followed by targeting to late endosomes. Cathepsins, a family of proteases, then progressively degrade Ii, leaving only a small fragment (CLIP) in the peptide-binding cleft. CLIP maintains to block the binding of peptides. Producing CLIP is the actual step that liberates MHCII αβ-dimers and, more importantly, this step removes the targeting information that the Ii cytoplasmic tail contains. After Ii processing HLA-DM, a chaperone protein, replaces CLIP from the peptide binding groove of MHCII with a peptide of 15-24 amino acids long[28]. These peptides are locally generated, by degradation of proteins present in the endo(lyso)somes. Peptides can either be formed from self-proteins or endocytosed and processed pathogens, the latter being most important in DC. Finally, the stable, peptide-bound MHCII (pMHCII) is presented on the DC cell surface.

Targeting to the endocytic pathway

It is essential that the $(\alpha\beta Ii)_3$ complexes are targeted to the endocytic pathway, because that is where pathogens and antigens are processed to peptides. For a long time it remained unclear whether targeting of MHCII happens directly from the trans-Golgi network (TGN) to the endosomes, or indirectly via the plasma membrane. A few years ago, two groups almost simultaneously provided

evidence that most of the $(\alpha\beta Ii)_3$ complexes are sorted via the plasma membrane to the endocytic pathway[29,30]. Both groups used RNA interference to knock down expression of AP-1 and AP-2. AP-1 and AP-2 are adaptor proteins that select the cargo for clathrin-coated vesicles. AP-1 is present on the TGN and on endosomes and selects cargo for direct transport from the TGN to endosomal compartments. AP-2 is found on the plasma membrane and selects cargo for endocytosis. Surface levels of Ii strongly increase if AP-2 is depleted from the cells. When AP-1 is depleted, only a small increase of Ii on the cell surface is noticeable[29,30]. These data suggest that $(\alpha\beta Ii)_3$ complexes are primarily delivered at the plasma membrane and then rapidly internalized for sorting to the endosomal system in an AP-2-dependent manner. Interactions between dileucine-based signals present in the cytoplasmic tail of Ii and AP-2 are necessary for endocytosis.

Invariant chain processing in maturation

In the past, some studies claimed to have shown that the degradation of Ii has a higher efficiency during the maturation process of DC. This would lead to a higher antigen loading and remove the restriction signal to the endosomes[31]. It was thought that Cystatin-C, a cathepsin S inhibitor, whose concentration is also decreasing upon maturation, regulates this Ii processing[31]. However, in knockout mice for Cystatin-C the processing of Ii was not altered and thereby the former statement is disproved[32]. Furthermore, other studies show no alterations in Ii processing upon maturation[20,33]. A conclusive study was performed by Ten Broeke *et al*. It was shown that the components of the medium in which DC grow influence Ii processing. Glutamine (Gln) is usually added to cell culture media as an essential nutrient. However, at temperatures of 37°C Gln is readily disintegrated, thereby leaving NH_4^+ in the medium. This decreases the pH in endosomes, thereby affecting the lysosomal enzymes required for Ii processing. NH_4^+ accumulation in Gln-containing medium inhibits the processing of Ii in endosomes and also affects MHCII degradation in lysosomes[26]. Experiments done to test Ii processing capacity and MCHII degradation should therefore use a medium with stable Gln (UltraGlutamine) or correct for these side effects of the medium. This finding also explains why previous studies found a higher degradation of Ii during maturation. Activating DC with LPS, when cultured with UltraGlutaime, showed no change in p10 degradation. In contrast, when NH_4Cl or Gln was present in the medium, degradation of Ii was severely repressed[26]. Thus, Ii processing is already very efficient in immature DC and therefore no changes in this processing occur during maturation.

MHC class II loading compartment

Where exactly does the MHCII loading with peptides take place? When exploring literature, it is often described that MHCII loading happens in a compartment termed MIIC (MHC II compartment). Compartments that contained MHCII and were similar to late endocytic organelles were termed MIIC, in order to distinguish them from other organelles of the endocytic pathway. Vesicles containing MHCII were termed CIIV (class II vesicles) and represented earlier endosomal structures. It was thought that MIIC and CIIV were specialized structures in the endocytic pathway, committed to MHCII production and function. However, it was shown that these MIICs were non-specialized multi vesicular bodies (MVB) or lysosomal compartments containing MHCII[34]. Nowadays, the term MIIC is a collective term for all MHCII containing compartments. Still, the CIIVs are characterized as distinct structures, without containing classical markers for endosomes and a role for CIIVs has been proposed in a late step transporting new pMHCII to the cell surface (Figure 4)[35]. The presence of MHCII-containing tubules was shown with immune-electron microscopy after treating a murine DC with LPS[36]. This study was extended with life cell imaging in murine BMDC where MHC II molecules were tagged with green fluorescent protein (GFP). Following LPS stimulation formation of lysosomal

tubules was visualized, and these tubules moved in a retrograde way to fuse at the plasma membrane[37]. Because of the role these DC tubes have in the late stages of pMHCII transport, they might correspond to CIIVs. However, such a relationship has not yet been proven[38]. Figure 3 provides an overview of the proposed transport route of MHCII. After synthesis and transport of $(\alpha\beta Ii)_3$ complexes to the cell surface, targeting to early endosomes occurs. Next, the MHCII dimers are formed and loaded with peptides. The exact location of these events is unknown, although it happens somewhere in the endocytic pathway where the pH is more acidic and HLA-DM is present. Then, pMHCII is transported to the plasma membrane, possibly via MHCII transport tubules and vesicles[38]. The details of pMHCII transport remain unknown.

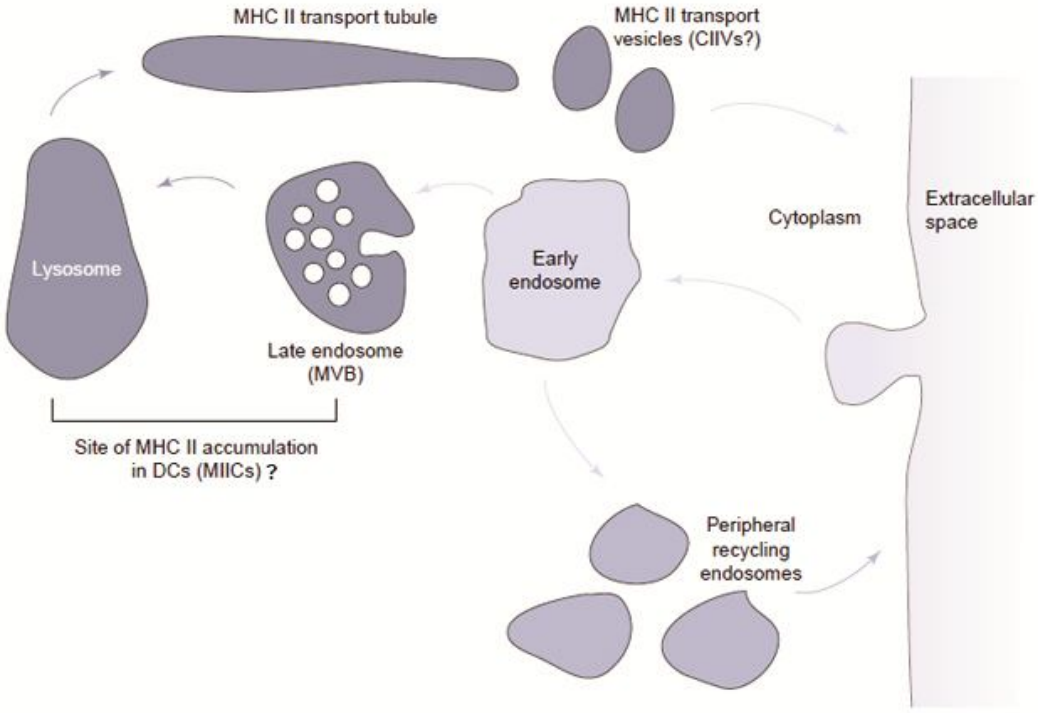

Figure 4. Schematic representation of the endocytic pathway for MHCII. Internalized proteins and other molecules will first enter the early endosome, and after transition through the late endosomes will reach the lysosome. Normally, degradation occurs in the lysosomes. Alternatively, sorting from early endosomes to peripheral recycling endosomes might occur, providing an escape-route back to the cell surface (more information: chapter three). MHCII is also internalized and via early endosomes targeted to late endosomes and lysosomes. Somewhere in this pathway does the peptide loading occur, but conclusive evidence is lacking. After observations made with EM, it seems likely that from lysosomes MHCII tubules occur that direct pMHCII to the cell surface. CIIVs are then intermediate structures. The light shades indicate earlier compartments, dark shades indicate late endosomal compartments. Adjusted from Chow *et al.*, 2005[38].

Endosomally stored MHCII

Some contradictions existed about the role of intracellular MHCII. As mentioned in chapter one, conventional DC first increase and then shut off the MHCII synthesis after a maturation stimulus[20,39]. Interfering with the biosynthetic pathway of MHCII production with drugs inhibits the high levels of MHCII on the cell surface, i.e. the main characteristic of mature DC. This is consistent with the theory that most of the cell surface MHCII in mature DC is newly synthesized during maturation[39]. On the contrary, in the steady state immature DC about 65% of MHCII is located at multi vesicular bodies (MVB) and the majority of these MHCII molecules are associated with intra luminal vesicles (ILV)[36]. Right after activation of DC, endosomal tubules are formed with the same rate that ILVs disappear[36]. This led to the theory that MHCII is stored at ILVs in immature DC and upon activation are sorted to

the plasma membrane. To study these contradictory theories, an mannosidase-II inhibitor (swainsonine) was used to create EndoH sensitive MHCII on the cell surface. EndoH removes the high mannose glycans present on newly synthesized glycoproteins. Proteins that have passed the Golgi and are therefore complexly glycosylated are resistant to EndoH. DC were activated by LPS stimulation in the absence or presence of swainsonine, in order to determine what the major source of stable MHCII on the cell surface is on activated DC. Afterwards, EndoH sensitivity of MHCII on the cell surface was measured. In the absence of swainsonine, only EndoH resistant MHCII reached the plasma membrane. If, after activation, MHCII is newly synthesized and swainsonine is present, EndoH sensitive MHCII should appear on the cell surface. After treating cells for 4 hrs with LPS and swainsonine ±57% of plasma membrane MHCII was EndoH sensitive and after 8 hrs this was ±76%. Furthermore, when ER-exit was inhibited with brefeldin A (BFA) no EndoH sensitive MHCII could be measured on the cell surface after treatment with LPS and swainsonine[40]. These results strongly indicate that the increase in MHCII on the cell surface after activation of DC is dependent on newly synthesized MHCII, and not derived from stored MHCII on MVBs. Most likely, pMHCII targeted to ILV at MVBs and lysosomes is degraded in immature DC, maintaining the low expression of MHCII on the cell surface[40].

The amount of information about the synthesis and transport of MHCII is ever growing. At this moment, we know that MHCII is first sorted to the plasma membrane in an AP-2-dependent manner and after that is targeted to the endocytic pathway. Here, cleavage of Ii releases MHC αβ-dimers and loading of the peptide-binding site occurs in assistance of HLA-DM. In immature DC, there is an internal pool of MHCII found on MVB and lysosomes. However, this internal pool does not contribute to the increased MHCII expression on the cell surface after activation. In immature DC, once MHCII has reached the lysosomes, it is probably degraded and not functional. In maturing DC newly synthesized MHCII reaches the endocytic pathway and is subsequently loaded with antigenic peptides and sorted to the plasma membrane. This probably happens via intermediate structures like transport tubules and MHCII vesicles. The exact site of peptide loading remains to be determined.

Chapter 3. Regulation of MHC class II by ubiquitination

In immature DC, part of the generated pMHCII is sorted directly to ILV and subsequently degraded in lysosomes. However, a portion of pMHCII is able to reach the cell surface. Interaction between pMHCII on immature DC and T cells are thought to play an important role in immune tolerance[41]. However, the half life of pMHCII in immature DC is short due to endocytosis. Upon maturation, synthesis of MHCII is upregulated and endocytosis of pMHCII is blocked, in order to create high levels of stable pMHCII on the cell surface. Therefore, mechanisms regulating these processes should be present. Convincing evidence has shown that ubiquitination plays an important role in regulating surface expression of MHCII.

Ubiquitination

In the late 1970s it was discovered that ubiquitin, a 76-amino acid peptide, could be tagged to a protein substrate. This is an ATP-dependent process and important during the breakdown of proteins. Currently, the cascade of events that mediate ubiquitin-conjugation to a protein substrate are unraveled (Figure 5). First, ATP is used to form a bond between the glycine residue of ubiquitin (Ub) and the cysteine residue of E1. In this way, Ub is 'activated'. Second, this activated Ub is transferred to E2. Third, this Ub-E2 complex is recruited by E3, an ubiquitin-protein ligase. E3 can specifically bind protein substrates and then facilitate the transfer of Ub from E2 to a lysine residue of the protein. Sometimes, Ub is first transferred to E3 and then to the protein substrate (not shown in Figure 5)[42].

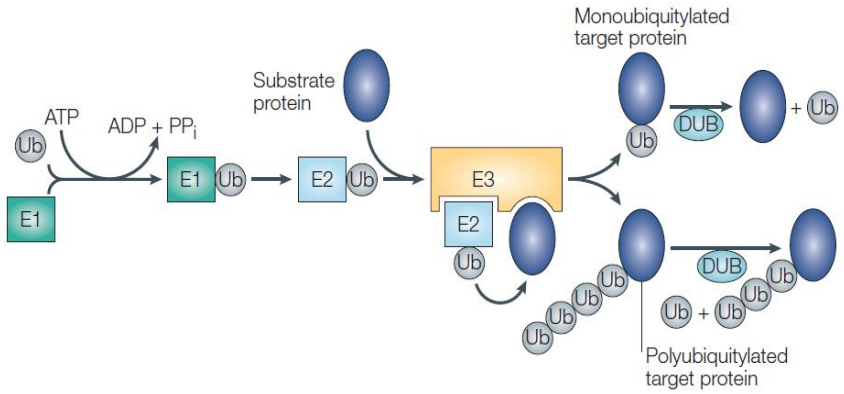

Figure 5. The ubiquitin-conjugation cycle. Ubiquitin is activated by E1, the activated Ub is transferred to E2. E3 recruits both Ub-E2 and the substrate protein, and facilitates the transfer of Ub to the substrate. Ub conjugated to substrates can be removed by DUBs (de-ubiquitination enzymes)[42].

Substrates can be conjugated with a single ubiquitin molecule, which is called monoubiquitination. The other option is the formation of an ubiquitin chain after initial tagging of one Ub, which results in a polyubiquitinated protein. Multiple mono-ubiquitination can also occur, when multiple lysine residues in substrate are conjugated with one Ub[43]. Ubiquitin molecules in a polyubiquitin chain are mainly linked through the lysine residues at positions 48 or 63 in ubiquitin (termed K48- and K63-linked polyubiquitination, respectively). Remarkable is that different types of polyubiquitin chains have different effects on the substrate. Tagging proteins with Ub can result in protein degradation by the proteasome, but can also modify the function of that protein, facilitate cell-surface expression and control gene transcription. K48-linked polyubiquitination, for example, targets substrates for degradation by the proteasome. K63-linked polyubiquitination on the other hand, modulates protein-protein interactions[44]. It has been shown that monoubiquitination is involved in down-regulation of receptors through targeting these receptors to the endo-lysosomal pathway[7].

Ubiquitination of MHCII

The first clue that ubiquitination is important in regulating MHCII expression was provided when c-MIR (modulator of immune recognition), an E3 ubiquitin ligase, was shown to ubiquitinate the MHCII β-chain (MHCII-β)[45]. Before this study, it was proposed that c-MIR belongs to the E3 family and has a function in the immune system[46]. MIR family proteins induce ubiquitination through binding of target proteins and ubiquitination of lysine residues in the cytoplasmic tail. c-MIR is a protein found in mice, and the mammalian E3 family proteins are called MARCH (membrane-associated RING-CH) proteins. This name comes from the catalytic domain of these proteins, which consists of a RING-CH domain. The homologue of c-MIR in humans is MARCH VIII. With this information, Ohmura-Hoshino and colleagues postulated that artificially manipulating the expression of c-MIR might modulate immune responses *in vivo*. Forced expression of c-MIR in BMDC inhibited antigen presentation. Furthermore, this could also prevent the onset of experimental autoimmune encephalomyelitis. After the observation that CD4 T cell development was severely impaired in transgenic mice with forced c-MIR expression, MHC levels were tested. MHCI levels were normal compared to WT mice. MHCII levels, however, were significantly lower in these transgenic mice. Because c-MIR was thought to be an E3 ligase, MHCII α- and β-chains were searched for possible lysine residues. Only MHCII-β contains one lysine residue in the cytoplasmic tail, at position 225. Mutating this lysine 225 into an arginine residue (K>R) resulted in no effect of c-MIR on the expression of MHCII. In control cells, where lysine 225 was not mutated, c-MIR was able to reduce MHCII expression (Figure 6). Further experiments were done to confirm that c-MIR actually ubiquitinates lysine 225 in the MHCII β-chain and that in the presence of c-MIR β-chain degradation was enhanced in WT compared to the K>R mutant. The last experiment was done to test if endocytosis of ubiquitated MHCII molecules is increased. Surface MHCII was labeled with FITC conjugated MHCII antibodies. The following chase experiment showed that cells with c-MIR rapidly endocytose MHCII, whereas control cells show stable levels of MHCII on the cell surface. It should be noted that this experiment does not actually test if endocytosis of ubiquitated MHCII is increased. It only shows that in the presence of c-MIR endocytosis is enhanced, suggesting that ubiquitination of MHCII is necessary for endocytosis. Together, these data provide evidence that MHC II is a target for c-MIR ubiquitination and that the down-regulation of MHCII surface expression depends on the presence of a single lysine in the β-chain cytoplasmic tail[45].

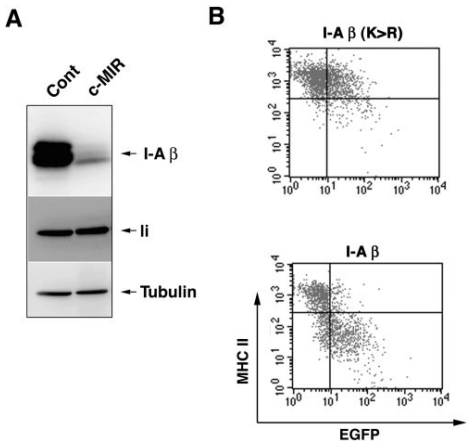

Figure 6. MHCII down-regulation by c-MIR. A. Protein samples from A20.2J cells (Cont) or A20.2J cells over-expressing c-MIR were analyzed after Western blotting with anti-β-chain Ab. B. MHCII expression on M12 C3 cells with WT (lower panel) or with mutated β-chains (K>R, upper panel). The cells were transfected with GFP-c-MIR[45].

After discovering that MHCII could be ubiquitinated in an overexpression experiment, the first thing to be done was to determine whether endogenous MHCII-β is also ubiquitinated under physiological conditions. For this purpose, BMDC and splenic DC were precipitated with α-ubiquitin antibodies. A ladder of proteins could be detected, corresponding to oligo-ubiquitinated MHCII β-chains with 1-5 ubiquitin molecules. Next, WT β-chain or mutant (K>R) β-chain was reintroduced in DC from MHCII β-chain deficient mice. Overall expression of MHCII-β was lower, nevertheless transduction with the

WT β-chain showed the same oligo-ubiquitin ladder. Cells transduced with MHCII β(K>R) showed no such ladder at all[27]. Furthermore, MHCII from MHCII β(K>R) primarily localizes at the plasma membrane in immature DC, instead of localizing at late endosomes or lysosomes, with a more then 20 fold increase. Ubiquitination is able to enhance MHCII degradation in lysosomes, and by doing so accumulation of MHCII at equilibrium is decreased. Ubiquitination is not only required for efficient MHCII endocytosis in immature DC, also in mature DC ubiquitination regulates endocytosis. This indicates that ubiquitination of MHCII is selectively down-regulated upon maturation, thereby enabling MHCII to accumulate at the plasma membrane[27].

In that same year, Van Niel and colleagues confirmed the data found in the previous two studies and provided more detailed information themselves. By using different antibodies against MHCII, it was possible to distinguish between Ii-bound, CLIP-bound of peptide-bound MHCII. Results show that MHCII-β is only ubiquitinated when MHCII is loaded with peptides. Thus, before ubiquitination can occur Ii processing is required[47]. LPS and other maturation stimuli can rapidly reduce the amount of ubiquitination of MHCII. This suggests that ubiquitination is strictly regulated and explains why MHCII in mature DC is stably expressed and has an increased half-life. Membrane proteins can be degraded after sorting into LVs at MVBs. Both endocytosis and sorting to MVBs is often triggered by ubiquitination of the cytoplasmic tail of these membrane proteins[48]. Using immunoelectron microscopy on ultrathin cryosections of immature DC made it possible to show that ubiquitination of MHCII-β is necessary for sorting at MVBs. If ubiquitination is blocked by mutating lysine 225, MHCII is transferred by the default pathway to the plasma membrane. Furthermore, ubiquitination is required for efficient endocytosis of MHCII[47]. The result of MHCII ubiquitination is intracellular retention of MHCII in immature DC. As mentioned in chapter two, MHCII-Ii complexes at the cell surface are predominantly endocytosed via interactions between dileucine-based signals in the cytoplasmic domain of Ii and the clathrin adaptor AP-2[29,30]. This is in line with the data that MHCII-Ii complexes are not ubiquitinated, because a dileucine-based signal for endocytosis is present.

MARCH I down-regulation

The studies described above provide evidence for a now generally accepted mechanism for regulating MHCII expression and transport. Shortly after these studies, it was shown that ubiquitination of MHCII also occurs in human monocyte-derived DC[49]. With an antibody specific for peptide-bound MHCII a ladder of polyubiquitinated β-chain was found. The most prevalent, however, was monoubiquitinated MHCII-β[49]. This was not unexpected, since monoubiquitination is known to target receptors to the endo-lysosomal pathway. It was impossible to find these polyubiquitination ladders with an antibody against MHCII bound with Ii, indicating that only mature, peptide-bound MHCII is regulated by ubiquitination.

At this point, the more interesting question about how ubiquitination of MHCII was regulated was proposed. Ohmura-Hoshino and colleagues show that c-MIR (MARCH VIII) overexpression leads to internalization of CD86 and MHCII. Furthermore, deleting MARCH I from mouse B cells promotes an increase in MHCII surface levels[50]. Because at least eight of these proteins of the MARCH family of ubiquitin E3 ligases are expressed in human DC, part of the regulation of ubiquitination was thought to be done by MARCH proteins. The next step therefore was to investigate the role of the different MARCH proteins in human DC. MARCH I through MARCH IX were tested and surface expression of MHCII was effected by MARCH I and, in line with expectations, by MARCH VIII. Further investigation

of MARCH I revealed that expression of MARCH I in mature DC could not be detected. Real-time quantitative PCR showed the down-regulation of MARCH I transcription upon LPS-induced maturation in DC (Figure 7). MARCH I favors the direct internalization of MHCII-peptide complexes in immature DC[49]. These experiments indicate that MARCH I is a major regulator of MHCII internalization and intracellular traffic. MARCH I down-regulation of in human DC results in acquiring a mature phenotype. Generating pMHCII in immature mouse BMDC is an efficient process. The ubiquitination of pMHCII by MARCH I, however, promotes their turnover[51]. Remarkably, only a very small fraction of the total MHCII molecules was ubiquitinated in DC[49]. The idea that MHCII is efficiently deubiquitinated after internalization, most likely in the late endosomes, seems to be supported by this finding. MARCH VIII transcription levels remained the same upon maturation, which might indicate that MARCH VIII, however capable of ubiquitinating MHCII, primarily has other functions in the cell. Therefore, it would be interesting to test if MARCH I and VIII are able to complement each other or cooperate in DC.

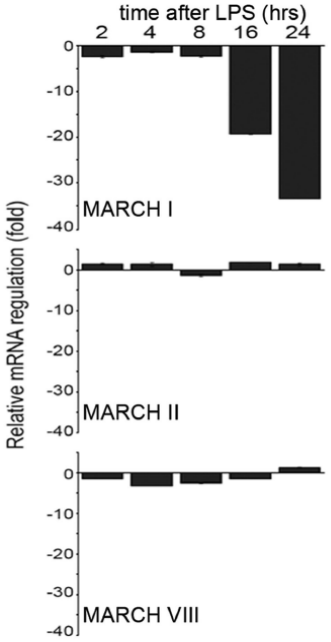

Figure 7. MARCH I down-regulation during MoDC maturation. Expression levels of MARCH I, II, and VII mRNA were measured after the indicated time of LPS stimulation by RT q-PCR[49].

Interestingly, the significant down-regulation of MARCH I only occurs 16 hours after LPS treatment (Figure 7), while after 2 hours the ubiquitination of MHCII was already strongly reduced. How can this be achieved? Probably, other regulatory mechanisms are involved in the short term decrease of ubiquitinated MHCII. For instance, other E3 ligases could be inactivated or deubiquitination enzymes (DUBs) could be activated. The data about c-MIR indicate that involvement of multiple members of the MARCH family is very likely. However, the possible role of DUBs in ubiquitination of MHCII remains to be investigated.

Are LPS-activated DC representative?

Almost all studies investigating the changes that occur in DC upon maturation, use LPS as a means of inducing this maturation. LPS is not the only agent that can be used, and certainly not the only one able to trigger maturation in the human body. It remained unclear if activating DC via different stimuli (e.g. different TLR ligands and non-TLR ligands) will lead to similar changes in the DC. More specifically, if MHCII expression, cell surface stability and ubiquitination are similar under all conditions. To this end, monocyte-derived human DC were activated with different ligands: LPS (TLR4), peptidoglycans (TLR2), dsRNA (TLR3), and even infection with an intracellular parasite. Regardless of the activation stimuli, the maturation in DC followed the same pattern of MHCII and CD86 upregulation, reducing MHCII ubiquitination and prolonged half-life of MHCII[52]. Stabilization of MHCII on the cell surface therefore seems to be a highly conserved step in all forms of DC activation. Thus, using LPS to activate DC and trigger maturation is equally good as using any other agent.

Ubiquitination in immature DC

In immature DC, pMHCII on the cell surface is rapidly endocytosed. Endocytosis is strongly driven by ubiquitination, as clearly stated above, however there also is a cytoplasmic di-leucine motif in the MHCII β-chain[53]. This di-leucine motif is probably present to drive aselective endocytosis. After

endocytosis, pMHCII encounters another possibility for ubiquitination driven sorting. Ubiquitinated pMHCII is sorted to ILV at MVB and thereby targeted for degradation in lysosomes, when MBV fuse with lysosomes. Sorting to ILV of ubiquitinated pMHCII is likely to be dependent on ESCRT (endosomal sorting complex required for transport) proteins, since ESCRT proteins recruit ubiquitinated membrane proteins at the endosomes/MVB[8]. Sorting to ILV blocks the receptor-function of MHCII and promotes degradation[54]. In immature DC, there is a constant production of MHCII which are loaded with self-peptides but rapidly internalized after expression at the plasma membrane. On the contrary, maturing DC should establish stable and high expression levels of pMHCII by abrogating the ubiquitination of MHCII. Newly synthesized MHCII is loaded with peptides from internalized pathogens. High pMHCII expression levels are, at least partly, achieved by down-regulation of MARCH I. Recently, CD83 has also been demonstrated to increase pMHCII expression by prevention of the interaction between MHCII and MARCH I[55].

Recycling of MHCII

Rapid internalization is the fate of pMHCII that reaches the cell surface in immature DC, however, the majority swiftly recycles back to the plasma membrane. Recycling pMHCII might exchange peptides in the early endosomes, which can happen in both a HLA-DM dependent or HLA-DM independent manner[56,57]. It is likely that this pool of recycling pMHCII is a significant part of the total endosomal and lysosomal pool of MHCII. Newly synthesized MHCII is associated with Ii when passing through the early endosomes, preventing the binding of antigenic peptides that might be present here. It has been suggested that recycling pMHCII could function as a means of increasing the repertoire of antigenic peptides that MHCII can bind and present to T cells[58]. Some peptides generated in the early endosomes will be degraded upon acidification of the endosomal compartments. In the absence of recycling pMHCII, only a specific group of highly stable peptides could be presented on to T cells. Since Ii-associated MHCII endocytosis occurs in an AP-2- and clathrin-dependent manner, as discussed in chapter two, it was investigated if pMHCII endocytosis depends on the same proteins. Remarkably, pMHCII endocytosis is independent of clathrin, AP-2, and dynamin. Internalized pMHCII can be found on tubular structures also containing Arf6 and Rab35[58]. These small GTPases are thought to be involved in clathrin-independent protein recycling, which is in line with the recycling of pMHCII. Through which mechanism pMHCII endocytosis is facilitated, and if there are separate mechanisms for endocytosis of recycling pMHCII and pMHCII targeted for degradation, remains to be elucidated.

At the moment, there is a theory further describing the role of recycling MHCII. This theory is based on the assumption that ‘new’ MHCII is primarily present in early endosomes and forms the recycling pool of MHCII and that ‘older’ MHCII is targeted to the later compartments for degradation (i.e. MVB and lysosomes). MHCII undergoes cycles of internalization and during every cycle there is a chance of escaping sorting to ILV and being transported back to the plasma membrane. However, this chance is minimal in immature DC because of effective ubiquitination. Since loading of MHCII is an enzymatic reaction, which will take place when MHCII, HLA-DM, and peptides are present, a problem with loading of self-peptides occurs. By what means can expression of self-peptides be kept to a minimum? There is no regulation in terms of peptide loading itself. However, self peptides are more abundant in the late endosomal compartments and therefore loading with self peptides mostly occurs on MHCII molecules targeted for degradation. When DC become activated, the upregulation of MHCII synthesis and abrogation of MHCII ubiquitination ensures that antigenic peptides present in

the early endosomal compartments are loaded onto newly synthesized MHCII and presented on the cell surface[39]. The earlier mentioned data that endosomally stored MHCII are not contributing to antigen presentation in activated DC are supporting this theory. Thus, DC are able to skew self peptide presentation and thereby prevent autoimmune responses during pathogen contact.

ITAM signaling

Despite the growing evidence that ubiquitination is involved in regulating pMHCII surface expression and trafficking in the cell, little information is known about the signal transduction pathways regulating pMHCII trafficking and antigen presentation. However, it has been shown before that the effector functions in neutrophils and DC are dependent on immunoreceptor tyrosine-based activation motif (ITAM) signaling[59,60]. An ITAM is a conserved four amino acid motif that is present in the cytoplasmic tails of certain receptors of immune cells. A tyrosine residue and two random amino acids are followed by a leucine or isoleucine residue (YxxL). Typically, two of these motifs are present and separated by 7-12 amino acids in the cytoplasmic tail[61]. After ligand binding to an ITAM-containing receptor the tyrosine residues are phosphorylated, leading to the formation of docking sites for other proteins that are involved in the signaling pathways of the cell. Integrin-mediated adhesion in DC can lead to the activation of Src family kinases, which will phosphorylate ITAMs in DAP-12 and Fc-*gamma* receptors (FcγR). FcγR are IgG receptors and able to bind the Fc region of IgG antibodies[62]. FcγR are important in recognizing and phagocyting IgG-bound pathogens. DAP-12 and FcγR become docking sites for recruiting the tyrosine kinase Syk, and in turn this kinase will phosphorylate several substrates involved in the formation of a signaling complex. SLP-76 and Vav proteins are essential components in this complex to initiate the cellular response that is associated with inflammation[63]. In this way, ITAM signaling is involved in activating the inflammation response in DC. On the other hand, ITAM signaling is also able to inhibit inflammatory responses in DC[64]. DC deficient of DAP-12 and FcγR show enhanced maturation and exogenous antigen presentation *in vitro* after TLR stimulation. Therefore, the precise role of ITAM signaling in DC during MHCII presentation and T cell priming remains unclear. Only recently, a new function of ITAM signaling in DC was found. ITAM signaling is required for the efficient priming of CD4 T cells, both *in vitro* and *in vivo*. Furthermore, ITAM signaling promotes the rescue of pMHCII once in the recycling pathway. DAP-12 and FcγR deficient DC fail to induce a proliferative response in CD4 T cells. ITAM signals are required for regulating the half life of stable pMHCII complexes at the plasma membrane. In the absence of ITAM signaling, pMHCII complexes are rapidly ubiquitinated and inefficiently recycled[63]. As a consequence, a higher amount of pMHCII in immature DC is targeted for lysosomal degradation resulting in a loss of the recycling MHCII pool.

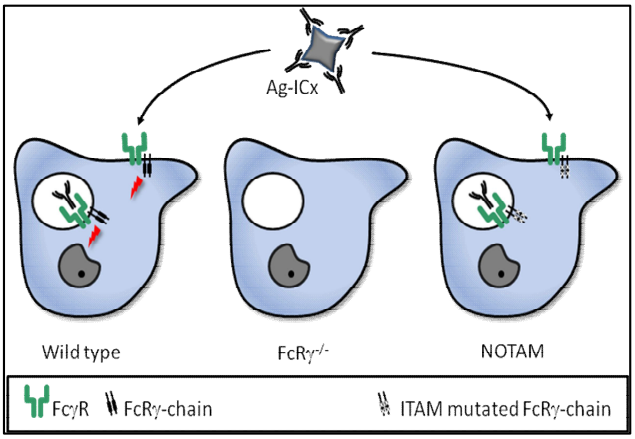

Figure 8. Mouse models to study the role of ITAM signaling in MHC class II presentation pathways. IgG-antigen complexes (Ag-ICx) can be internalized by wild type and NOTAM mice. In FcRγ knockout mice, there are no FcRγ on the cell surface and therefore internalization of Ag-ICx and ITAM signaling are absent. In wild type BMDC ITAM signaling is normally functioning, while in the NOTAM mice ITAM signaling is perturbed (group of Leusen).

DC express four types of FcγR, three of which are activating receptors and associated with a γ-chain[62]. In the above mentioned study, this γ-chain is knocked out ($FcR\gamma^{-/-}$) in order to study the role of ITAM signaling. However, if the γ-chain is knocked out, there is no surface expression of the three activating FcγR. In these knockout mice the specific activating FcγR-mediated uptake of IgG-immune complexes (IgG-IC) is abrogated, however, soluble IgG-IC can still be taken up by the inhibitory FcγRIIB. By generating a more subtle mutant in which only the ITAM signaling in the γ-chain is abrogated, more specific data can be obtained (Figure 8). These mice, called NOTAM mice, have the unique feature that only the γ-chain signaling is perturbed while DC are still able to stabilize the expression of FcγR on the cell surface. In contrast to $FcR\gamma^{-/-}$ DC, internalization of IgG-IC by NOTAM DC is not fully abrogated, however, it is less efficient than by WT DC (unpublished data of Jeanette Leusen and colleagues). Strikingly, presentation of IgG-IC-derived antigens via MHCII or cross-presentation via MHCI was fully abrogated. At the moment, further experiments with NOTAM BMDC are performed and the data that are being generated will provide more insight into the signaling pathways underlying antigen presentation.

Discussion

Integrating all the information from the previous chapters leads to the formation of a model combining MHCII synthesis, transport, ubiquitination, surface expression, and the differences between immature and mature DC (Figure 9). This model also summarizes the main content of this thesis.

In the ER new MHCII are synthesized and complexed with Ii. MHCII-Ii is then transported through the Golgi to the plasma membrane. Here, AP-2 dependent MHCII-Ii endocytosis targets the complexes to the endocytic pathway. This process is driven by sorting signals in the cytoplasmic tail of Ii. Once in the endosomes, Ii is gradually degraded in a stepwise manner, resulting in Ii-free MHCII, which is no longer targeted to the endocytic pathway. The remaining CLIP occupying the peptide binding cleft of MHCII is then substituted by peptides present in the endosomal compartments with the aid of HLA-DM. This exchange is equally efficient in both immature DC and mDC. In immature DC however most of the newly formed pMHCII complexes are rapidly ubiquitinated, driving the active sorting of pMHCII to ILV of MVB. Probably, interactions with ESCRT proteins are involved in this process. Some of the new pMHCII complexes escape the sorting to ILV and are able to reach the cell surface of immature DC. Now, ubiquitination of cell surface pMHCII increases endocytosis and creates a second opportunity for sorting pMHCII to ILV at MVB. Endocytosis of pMHCII is independent of AP-2, clathrin, and dynamin. Sorting MHCII to ILV consequently leads to lysosomal degradation and MHCII cannot be rescued by recycling back to the plasma membrane via recycling endosomes. MHCII is normally loaded with self peptides in immature DC. Due to rapid internalization and degradation, expression of these self peptides can be kept to a minimum. The few MHCII-self peptide complexes that do reach the cell surface are proposed to play a role in immune tolerance and form a pool of recycling pMHCII. After several rounds of recycling, recycling pMHCII is also targeted to ILV[40].

In maturing DC MHCII synthesis is temporarily enhanced and ubiquitination of MHCII is simultaneously prevented. Now, MHCII is no longer sorted to ILV for degradation. In maturing DC, newly synthesized and recycling MHCII are likely to be loaded with antigenic peptides generated from phagocytosed pathogen. pMHCII are transported from endosomal membranes to the plasma membrane via MHCII transport tubules and vesicles. A portion of pMHCII might still be transferred to ILV independent of ubiquitination, however, this is a nonselective process. It has been shown that MHCII may also be transported from the MVB or lysosomal delimiting membrane to the plasma membrane, in order to present peptides generated in late endosomal or lysosomal compartments. DC that completed the maturation process shut off MHCII synthesis. In this way, the cell surface of mature DC is primarily covered with MHCII synthesized and subsequently loaded with peptides generated during the contact with pathogens.

How can we fit the role of ITAM signaling in regulating pMHCII levels at the cell surface? ITAM signaling would most likely cause changes in the ubiquitination of MHCII, since ubiquitination is essential in regulating MHCII levels. One plausible explanation is that ITAM signaling leads to phosphorylation of the cytoplasmic tail of MHCII-β, near the site of lysine 225. This modification could block the effective binding of E3 ligases to MHCII-β, thereby stabilizing pMHCII on the cell surface (Figure 10). It would be relatively easy to test this, by measuring if MHCII-β is phosphorylated in immature DC. Another possibility is that ITAM signaling activates kinases that phosphorylate

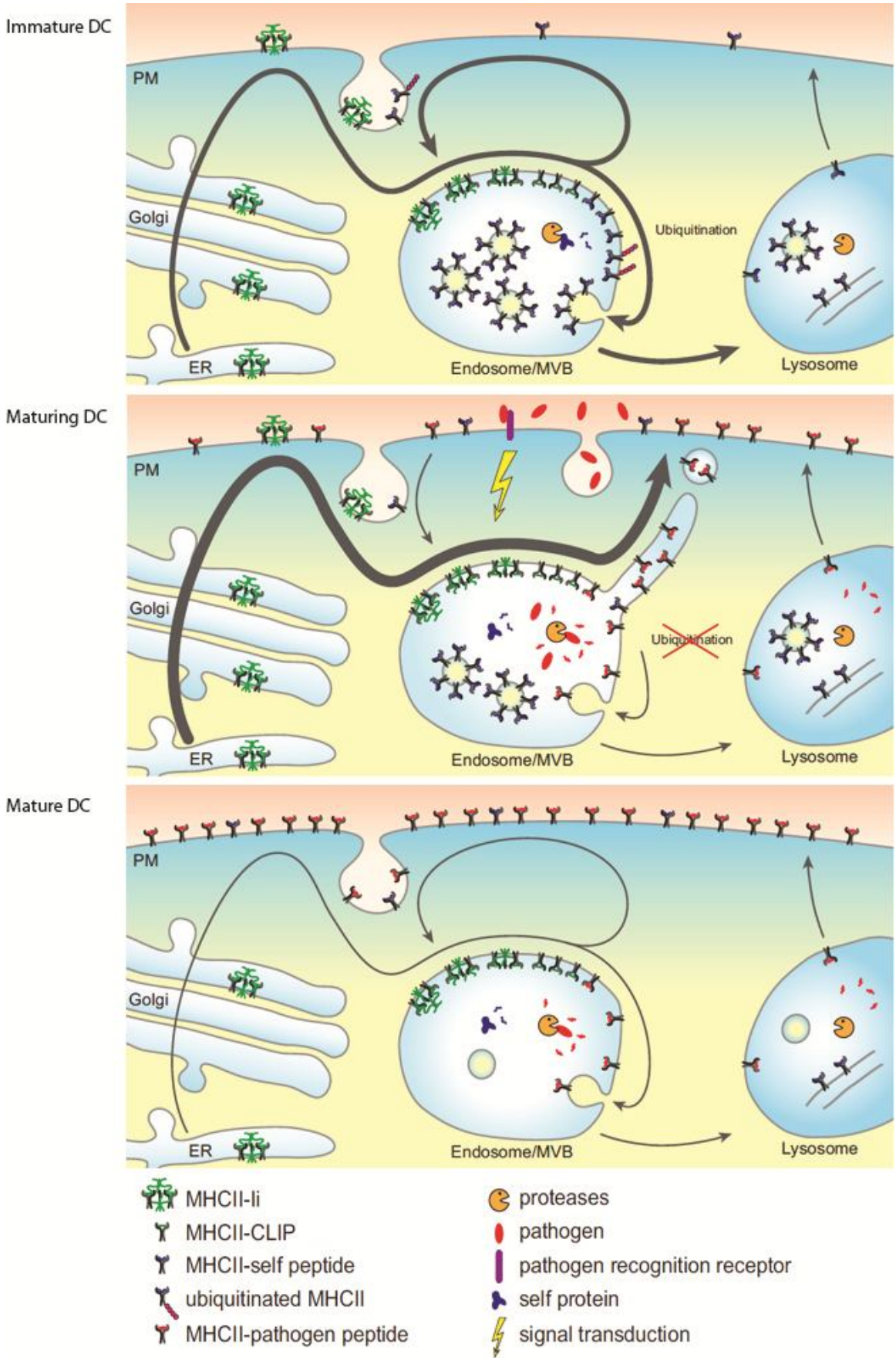

Figure 9. Model for MHCII traffic in DC. Processes are explained in the text. Thickness of arrow lines indicate efficiency of the different steps. In maturing DC MHCII synthesis is temporary upregulated[40].

certain DUBs or E3 ligases. Phosphorylation of these enzymes will alter their activity. Phosphorylation of DUBs would increase their deubiquitination activity and subsequently ubiquitin tags from MHCII will be removed, stabilizing MHCII levels at the cell surface. However, currently no DUBs are known to be involved in regulation of MHCII expression. Thus, an important future direction for research would be to test the influence of known DUBs on MHCII expression. If one or more DUBs are found to be involved, it could be tested if ITAM signaling increases the activity of these DUBs. In the same way, the effects of ITAM signaling on E3 ligases like MARCH I should be tested. Besides ITAM signaling, other signaling pathways might also be involved in regulation of MHCII expression in DC. ITAM signaling is probably only the tip of the iceberg of intracellular signals present in maturing DC. Understanding these complex pathways and their role in regulating ubiquitination will greatly increase our knowledge of antigen presentation.

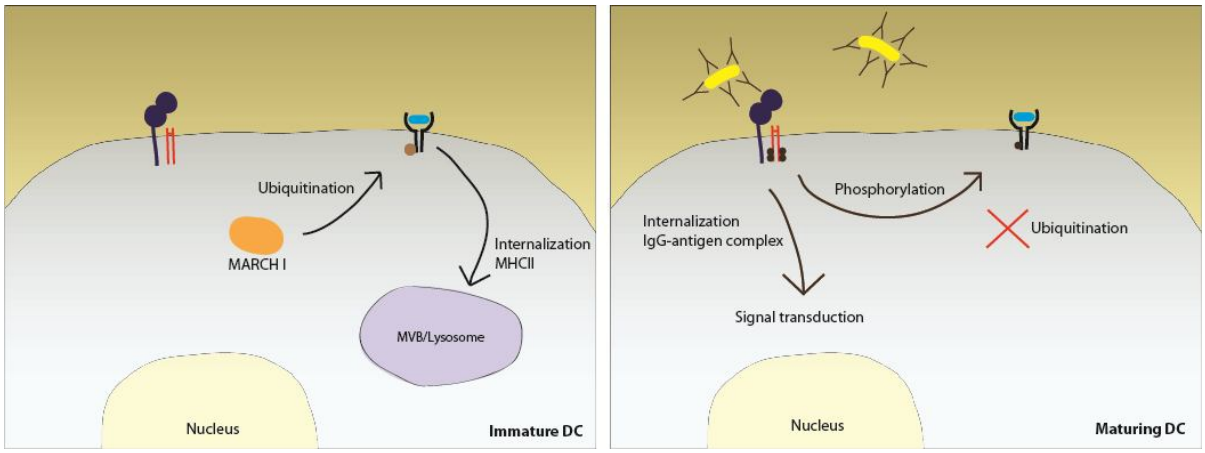

Figure 10. Model for the role of ITAM signaling in regulating ubiquitination of MHCII. In immature DC there is no ITAM signaling, since the FcγR is not stimulated. Now, MARCH I can facilitate ubiquitination of pMHCII at the cell surface, leading to internalization of MHCII and subsequent degradation in MVB/Lysosome. In maturing DC, FcγR are stimulated, in this case by antigen-immune complexes, leading to the phosporylation of the γ-chain. This initiates the internalization of the IgG-antigen complex, signal transduction to start the maturation, and rapid phosphorylation of the MHCII β-chain. The phosphate-group blocks the ubiquitination of MHCII, resulting in stable MHCII on the cell surface.

Most likely, ubiquitination is not the only process that regulates MHCII expression, since the transport of MHCII in the cell is such a complex process. Fully understanding the transport of MHCII would also result in ways to regulate MHCII expression. If, for example, the transport of pMHCII complexes could be enhanced, this would result in better antigen presentation. MHCII containing lysosomal tubules are present in maturing DC. In immature DC, however, it was impossible to visualize these structures with EM[37]. This could either mean that MHCII tubules are not formed in immature DC or that they are less pronounced and therefore difficult to visualize. With specific markers for these tubules, it might be possible to exclude one of the two possibilities using confocal microscopy. At this point, it is unknown how endocytosis of pMHCII occurs and which proteins are involved in the formation of endocytic vesicles. It could be tested if pMHCII complexes can bind other proteins known to be involved in endocytosis. Besides that, it would be interesting to test the current hypothesis about recycling pMHCII and their function in skewing self-peptide loading.

Ultimately, intensive research might provide us with means to modulate antigen presentation, preferably in specific subsets of DC. Knowledge about the differences in DC subsets could lead to stimulating MHCII expression in the right subset of DC, thereby activating a specific immune

response. Stimulating the presentation of tumor-antigens on MHCII could improve cancer treatment in patients and moreover, might even result in 'tumor memory' if memory T cells are generated. Recurring tumors cells could then be effectively killed by the immune system and a lifelong protection against these cells acquired. Furthermore, in all other diseases and infections where the adaptive immune system is involved, regulation of MHCII expression and antigen presentation will be of great value. Alternatively, drugs could be searched or designed that are able to increase or decrease the half-life of pMHCII on the cell surface of DC. Such drugs could be applied for enhancing efficient vaccine antigen presentation or on the other hand reduce self-antigen presentation in autoimmune diseases. Thus, future directions for research should be focusing on fully understanding antigen presentation in DC in order to modulate this process for the treatment of a wide range of diseases.

Acknowledgements

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Appendix I
Introduction to practical part

Part of this thesis was to perform experiments in the immunology laboratory, in order to learn about the techniques used in immunology research and to experience what research is all about. Together with another student, Lione Willems, I performed these experiments within the group of Jeanette Leusen. At the interface between immunology and cancer research, experiments are done to investigate if mAb cancer therapy could be improved by using IgA antibodies instead of IgG. Furthermore, the previously mentioned NOTAM mice are used to explore the role of ITAM signaling in MHCII presentation in DC in order to possibly enhance mAb cancer therapy. The experiments and results are described in Appendix II-VI.

A technique frequently used in the immunology laboratory is fluorescent activated cell sorting (FACS). After incubation of cells with fluorescently labeled Abs, the fluorescence of each individual cell is measured. Cells can also be sorted according to the fluorescent signal. Among others, FACS is used to determine the phenotype of cells and to determine the binding of antibodies to specific cells (for instance to compare the binding of IgA antibodies to tumor cells with IgG antibodies). Mean fluorescent intensity is determined by the amount of bound antibodies and corresponds to the expression of the measured antigen. Fluorescent antibodies are also used in confocal microscopy, where colocalization of proteins can be visualized.

Antibody-dependent cell-mediated cytotoxicity can be measured by radioactive assay. Tumor cells are loaded with a radioactive label, for example ^{51}Chrome, and incubated with specific antibodies. Effector cells, for example neutrophils, are added, which will recognize antibody-bound cells through their Fc receptors and kill them. Radioactive label is released from cells that are killed. Radioactivity in the supernatant corresponds to the amount of killed cells.

To study the degradation of immune complexes after uptake by different types of DC SDS-PAGE and Western blot are used. mAb therapy, on the other hand, can best be studied in mouse models. Bioluminescence imaging (BLI) can be used to follow tumor growth longitudinally. Tumor cells expressing luciferase are injected into mice and will spread throughout the body and form tumors. Injecting the substrate for luciferase, luciferin, results in the emission of light by these cells and the light emission is measured. This shows the location and size of the tumors. There are no results of the bioluminescence imaging experiment in the appendix, since we did not perform the experiment ourselves.

The range of techniques used in this laboratory show that the experiments generate information at different levels, from intracellular protein degradation to tumor therapy in mice. I have learned that this is important, in order to thoroughly understand a process and also to study the applications. In this way, a broad yet detailed view of a problem or process is generated. After performing these experiments, I think that conducting research at multiple levels is most effective.

Appendix II
Antibody-dependent cell-mediated cytotoxicity assay

Introduction

Effector cells of the immune system can actively lyse target cells that have been bound by specific antibodies. This mechanism is called antibody-dependent cell-mediated cytotoxicity (ADCC). It can be induced *in vitro* by adding mAb and mouse blood to a tumor cell line. The blood contains neutrophils (PMNs), which act as effector cells. They recognize mAb-coated cells and kill them.

ADCC assays are done with the human carcinoma cell line A431, which endogenously expresses high levels of EGFR. Here we will test whether ADCC can also be induced in a mouse pre-B cell line (Ba/F3) which is transfected with EGFR and expresses the EGFR on the cell surface (Ba/F3-EGFR). Both cell lines have pros and cons for their use in cancer research. The A431 cell line is human of origin and therefore a better model for human cancers, but it can only be used in immunodeficient mice. No correct adaptive immune responses can be generated in these mice. In contrast, Ba/F3 cells are mouse cells and grow in immunocompetent mice. Generation of adaptive immune responses is still possible in these mice.

The anti-EGFR mAb Cetuximab was added to the cells to induce ADCC. Mice were given PEG-G-CSF, which is recombinant G-CSF (granulocyte-colony stimulating factor) coupled to polyethylene glycol (PEG). This increases the percentage of PMNs in the blood through recruitment from the bone marrow and therefore the ADCC activity.

Tumor cell lysis is measured by the release of Cr to the supernatant. First, cells are incubated with radioactively labeled chrome (^{51}Cr), which is taken up by the cells. Secondly, mAb Cetuximab, binding EGFR on the target cells, is added. Finally, the cells are incubated with blood containing PMNs and the release of ^{51}Chrome is measured, which corresponds to the amount of cells killed by PMNs. To measure the amount of PMNs in the blood of the mice, fluorescence activated cell sorting (FACS) analysis is used. The percentage of PMNs in the blood is determined by staining of whole mouse blood with antibodies against surface molecules GR1 (expressed on PMNs and on a subpopulation of monocytes) and CD11b (expressed on both PMNs and monocytes). PMNs express both markers and therefore appear as double positive (CD11b+GR1+) cells.

Materials & Methods

^{51}Chroom ADCC assay

- Collect Ba/F3-EGFR pre-B cells
- Count tumor cells and wash cells with PBS
- Collect pellet in 80 µl PBS/ 1x10^6 cells
- Label 1x10^6 cells with 100µCi ^{51}Cr, 2h in an incubator at 37^{o}C, 5% CO_2
- Plate out Cetuximab (1mg/ml) in 96 wells plate in concentrations 0.1, 1, 10 µg/ml, dilute in medium to 125 µl (in triplo)
- Use 5% triton as maximal release, 150 µl /well
- Use culture medium as minimal release, 150 µl well
- Add mouse blood to plate (in triplo):

    - E:T ratio 25µl blood to $5x10^3$ Ba/F3-EGFR cells
    - E:T ratio 12,5µl blood to $5x10^3$ Ba/F3-EGFR cells
    - E:T ratio 6,25µl blood to $5x10^3$ Ba/F3-EGFR cells
- Wash away excess ^{51}Cr by washing 3 times with 10ml culture medium/ $1x10^6$ cells
- Assume that you lose 10% cells during washing, collect pellet in 9ml culture medium/ $1x10^6$ cells
- Use 50 µl cell suspension/well = $5x10^3$ Ba/F3-EGFR cells
- Incubate ADCC for 4h in an incubator at 37°C, 5% CO_2
- Collect supernatant with a skatron harvesting system
- Measure activity in a cobra counter, protocol 4

- Calculate % lysis:

$$\frac{(\text{exp count- min release})_{x100}}{(\text{max release- min release})}$$

FACS analysis

- Take 20 µl blood in two tubes (1 unlabeled, 1 labeled sample)
- Add 50 µl anti-mouse Ly-6G-FITC (Gr-1) diluted 1:100 in FACS buffer
- Add 50 µl anti –mouse CD11B-APC diluted 1:100 in FACS buffer
- Incubate 20 min at room temperature (RT)
- Lyse erythrocytes with BD FACS lysing solution (dilute 1/10 in water)
- 950 µl per tube, incubate 10 min at RT
- Spin tubes 1800 rpm, 3 min
- Discard supernatant, resuspend pellet in FACS buffer
- Measure with FACS Calibur

Results

The amount of PMNs in the blood was ±24%, as measured with FACS analysis. We measured very low radioactive signal with the cobra counter in all conditions. The positive control (triton) gave good results, indicating that the labeling of the cells was optimal.

Discussion

We observed no killing of Ba/F3-EGFR cells by PMN in this assay. One possible explanation is that the amount of PMNs in the blood was only ±24%, which is very low in mice injected with PEG-G-CSF. Normally the amount of PMNs without stimulation is about 15% and after injection with PEG-G-CSF it is 60-70%. The reason for the low PMN percentage is unknown and the experiment should be repeated with mice newly injected with PEG-G-CSF. Another explanation is that perhaps Ba/F3-EGFR cells cannot be killed by mouse PMNs. EGFR expression by Ba/F3-EGFR cells may not be high enough for potent killing by PMN. Ba/F3-EGFR cells are more sensitive to ^{51}Cr because around 50% of the cells is lost after labeling compared to 10% loss with other cell types. The experiment could be repeated with newly transformed Ba/F3 cells in which the expression of EGFR might be higher. If transformation of Ba/F3 cells is done in other conditions, sensitivity to ^{51}Cr labeling might be decreased.

Appendix III
Fluorescence activated cell sorting (FACS)

Introduction

All mAbs currently used in cancer immunotherapy are of the IgG isotype. However, killing tumor cells is more effective *in vitro* by IgA mAbs. In this experiment the binding of Cetuximab (IgG1 EGFR Ab) to EGFR is compared with the binding of the IgA1 and IgA2 variant of Cetuxiumab. This is evaluated in the human carcinoma cell line A431 and in mouse pre-B cells (Ba/F3 cells) transfected to express this receptor (Ba/F3-EGFR). As negative control normal Ba/F3 cells are used.

Increasing concentrations of the mAbs were added to the cells, and after incubation and a washing-step the mAbs were detected with an α-Kappa-Light Chain Ab. This antibody is labeled with biotin and mixed with a fluorescent conjugate (SA-APC). Fluorescence is measured with fluorescence activated cell sorting (FACS) and is related to the levels of mAb bound to EGFR.

To test whether EGFR Abs and EGF bind to the same epitope, the competition for binding to EGFR between EGFR mAbs and EGF ligand was tested. A constant concentration of ligand and increasing concentrations of mAb were added to these cells. As a control, increasing concentrations of EGF and a constant concentration of mAb were added.

Materials & Methods

Methods

- Collect and count cells of interest, use $1x10^5$ cells/ sample
- Wash the cells in FACS buffer (fb)
- Add Abs diluted in fb to the cells, 15 µl/sample, incubate 30' at RT
- Wash away unbound Ab with fb; Add 100 µl of fb to the cells, spin down 1500 rpm 3', discard the supernatant.
- Add secondary Abs diluted in fb to the cells, 15 µl/ well, incubate 45' at RT.
- Wash away unbound Ab with fb
- Add 150 µl fb/ well and measure on a FACS

Materials

- A431 cells
- Ba/F3-EGFR cells
- Ba/F3 cells
- α-EGFR Abs:
- Cetuximab, human IgG1-α-EGFR
    - hIgA1-EGFR, Gift from Thomas Valerius, Kiel, Germany
    - hIgA2-EGFR, Gift from Thomas Valerius, Kiel, Germany
    - Fb, PBS with 0.1% BSA and 0.1% sodium azide
- SA-APC, eBioscience, 17-4317-82, 38D use 1:100
- aKappa-LC-biotin, Southern biotech, 9230-08, use 1:100

Results

After FACS analysis graphs like Figure 11 were obtained. These two examples correspond to the addition of different concentrations of IgG Cetuximab to A431 cells. Cells showing fluorescence are measured. Fluorescence lower than the cut-off (10^3 APC-A) is considered to be background signal, probably due to aspecific binding of α-Kappa-Light Chain and SA-APC. In the sample of Figure 11a no mAb was added, resulting in just a background signal. A peak higher than the cut-off (in quadrant P2) is equal to the levels of mAb bound to EGFR. When more mAb is bound, the peak will shift to the right. Figure 11b is the result of adding 0.02 µg/mL Cetuximab. The number of events and the percentage of cells with bound mAb are shown in row P2 of the tables.

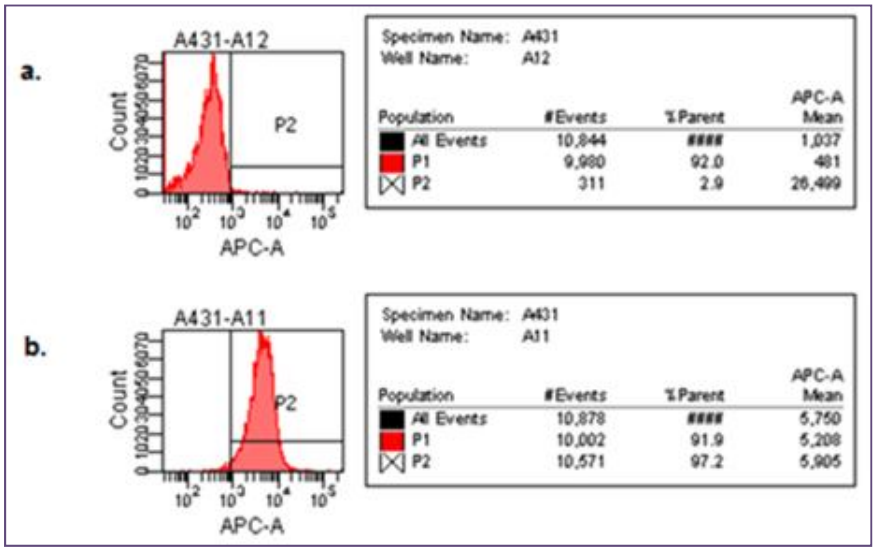

Figure 11. FACS analysis graphs of A431 cells. (A) A431 cells without mAb treatment. (B) A431 cells treated with 0.02 µg/mL Cetuximab.

In Figure 12, fluorescence (MFI, mean fluorescent intensity) is plotted against the mAb concentration for the A431 and Ba/F3-EGFR cell lines. An increase in fluorescence is seen due to an increase in the concentration of the mAbs. IgG and IgA1 have a similar affinity for EGFR in A431 and Ba/F3-EGFR cell lines (Figure 12a and 12b). Binding of IgA2, however, is lower. Moreover, fluorescent intensity is 10 times lower in Ba/F3-EGFR cells than in A431 cells indicating lower EGFR expression.
The effect of competition between EGF ligand and mAb in Ba/F3-EGFR cells is shown in Figure 12c. By increasing EGFR mAb concentration similar increase in fluorescence was detected in samples with and without EGF. In Figure 12d the mAb concentration was kept constant and the concentration of EGF ligand was increased, resulting in a decrease in fluorescence indicating a reduced binding by the EGFR Ab.

The same experiment is also done with an α-IgA-PE Ab instead of α-Kappa-Light Chain. In this way it was possible to determine whether the lower fluorescence of IgA2 was the result of lower binding to EGFR or a different recognition by α-Kappa-Light Chain to IgA2. Figure 12e shows that both secondary detection antibodies α-Kappa-Light Chain and α-IgA-PE show lower staining with IgA2 compared to IgA1. This is caused most likely due to a secondary Ab effect, maybe caused by glycosylation differences between IgG1, IgA1 and IgA2. IgA binding on Ba/F3-EGFR cells showed a sudden drop in the fluorescence at lower concentrations. The reason for this is not known and therefore the assay should be repeated.

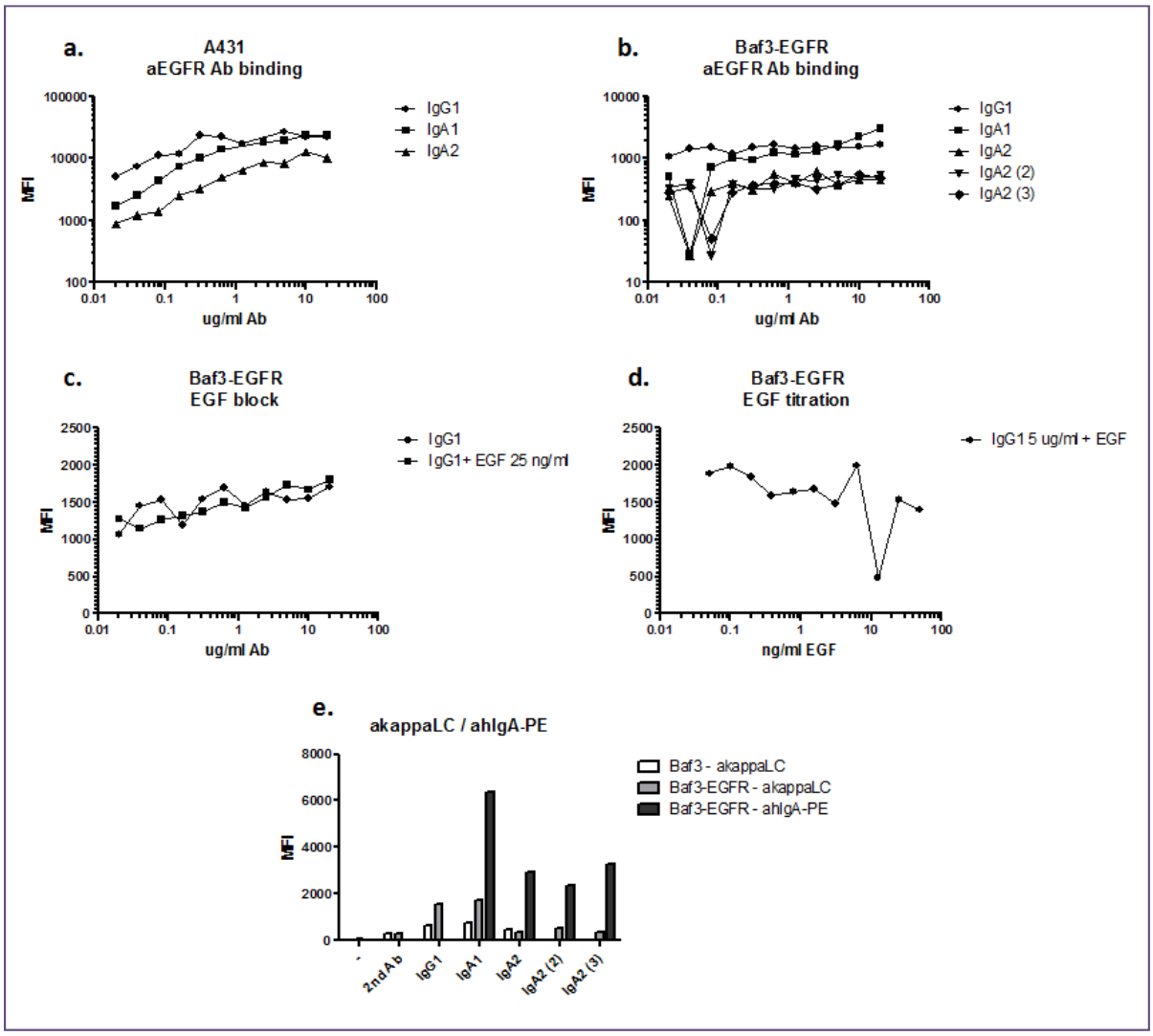

Figure 12. Mean fluorescent intensity of different mAb. (A) Binding of IgG, IgA1 and IgA2 to EGFR in A431 cells. (B) Binding of IgG, IgA1 and IgA2 to EGFR in Ba/F3-EGFR cells. (C) Binding of IgG to EGFR with or without 25 µg/mL EGF ligand. (D) Binding of 5 µg/mL IgG to EGFR with increasing concentrations of EGF ligand. (E) Fluorescent intensities measured using α-Kappa-Light Chain or α-IgA-PE.

Discussion

This experiment shows IgG1, IgA1 and IgA2 EGFR Abs all bind to both A431 and Ba/F3-EGFR cells. IgA2 binding is lower than IgA1 as detected by two different secondary antibodies. IgA1, therefore, might be more potent in cancer therapy. To see whether EGFR antibodies bind to the same epitope as EGF, a competition assay was performed on Ba/F3-EGFR cells. Adding high concentrations of EGF did not influence the binding of IgG, indicating that there is no competition between EGF and IgG. However, the second competition assay showed opposing results. A decrease in IgG affinity was observed when adding increasing concentrations of EGF, suggesting competition. However, the decrease in affinity is very minimal and probably not significant. The assay should be repeated with A431 cells, which express much higher levels of EGFR, to see if the decrease in affinity was significant or if there is no competition between EGF and EGFR IgG. While the affinity of EGFR Abs to EGFR is the same on all cell types, in Ba/F3-EGFR cells the expression of EGFR is 10 times lower than in A431 cells. Alternatively, EGFR conformation in the membrane on Ba/F3-EGFR cells may be different from the conformation on A431 cells, which may influence EGFR Ab binding.

Appendix IV
Immune complex binding and degradation assay

Introduction

FcγR are crucial for the immune system in recognizing IgG antibodies. In mice, DC express all four types of FcγR. FcγRI, FcγRIII and FcγRIV are associated with the γ-chain present in the plasma membrane. The γ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM) domain and generates activating signals in the cell after binding of IgG to the FcγR. FcγRII, on the other hand, is not associated with the γ-chain (Figure 13). The cytoplasmic tail of this receptor contains an immunoreceptor tyrosine-based inhibition motif (ITIM) and gives an inhibitory signal to the cell after binding the ligand.

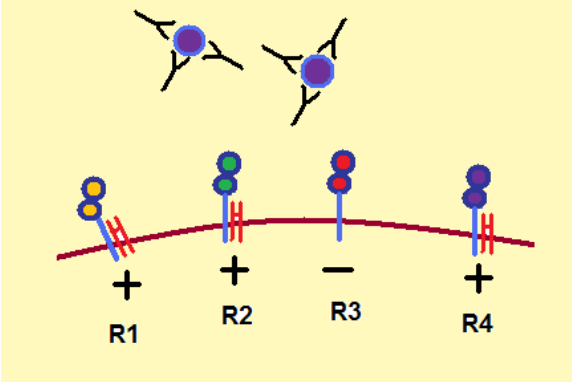

Figure 13 . Four types of FcRγ receptors. FcγRI, FcγRIII and FcγRIV are associated with the γ-chain present in the plasma membrane. FcγRII is not associated with the γ-chain and the only receptor generating an inhibitory signal.

FcγR are required for efficient presentation of immune-complex-derived antigens as shown by FcRγ-chain knockout mouse ($FcγR^{-/-}$). However, due to missing of the γ-chain these mice lack surface expression of FcγRI, FcγRIII and FcγRIV on DC. In these knockout mice the specific receptor-mediated internalization of IgG-bound antigens is also blocked, making it difficult to interpret the data. To deal with this problem, NOTAM mice have been developed recently. In these mice the intracellular signaling via ITAM is abrogated by mutations in the ITAM motif. The mutant γ-chain is still able to provide surface expression of all FcγRs.

Here, we cultured bone-marrow derived DC from three types of mice (wildtype (WT), $FcRγ^{-/-}$ and NOTAM). Phenotyping of DC was performed to check the quality of the DC. We investigated binding and uptake of immune complexes. We used immune complexes made of an IgG antibody and the Alexa488-labeled antigen ovalbumin (OVA-IC). To distinguish between binding and uptake of IC and uptake only we used Trypan Blue (TB) to quench fluorescence. Furthermore, a degradation assay was done to measure the degradation of OVA after IC uptake in DC. Proteinase K (ProtK) was used to remove all the OVA bound to the cells (but not internalized), to be able to distinguish between internalized OVA and OVA bound to the surface. Samples were analyzed with a SDS-PAGE gel and Western Blotting.

Materials & Methods

(Protocols can be found on p. 45-47)

- DC were derived from the bone marrow of WT, $FcRγ^{-/-}$ and NOTAM mice after culturing bone marrow with GM-CSF for seven days (see Protocol 1)
- FACS analysis to phenotype the DC
- Counting of DC

- Immune complex binding assay (Protocol 2): DC of different mouse genotypes and different concentrations of OVA-IC were incubated with or without TB
    - Fluorescence was measured with FACS to determine if OVA-IC is internalized
- Degradation assay (Protocol 3): DC were incubated with OVA or with OVA-IC at different temperatures and for different times, and with or without ProtK. Cells were lysed and samples run on a SDS-PAGE gel
    - OVA fluorescence was measured with Typhoon Variable Mode Imager
    - Western blot (Protocol 4): samples from SDS-PAGE were blotted on nitrocellulose blot and frozen for later analysis.

Results

Phenotyping

The amount of living cells is lower in FcRγ$^{-/-}$ and NOTAM mice compared to WT DC (Figure 14a). Furthermore, these cells are slightly more activated than WT DC, as can be seen by the higher percentage of CD40+/CD86+ cells (Figure 14b) and a higher MHC-I and MHC-II expression (Figure 14c). FcγR expression is as expected (Figure 14d): FcγRI, III and IV expression in NOTAM mice is similar to the expression in WT mice. FcRγ$^{-/-}$ DC have decreased expression of all three of these receptors; the differences are the least with FcγRIII.

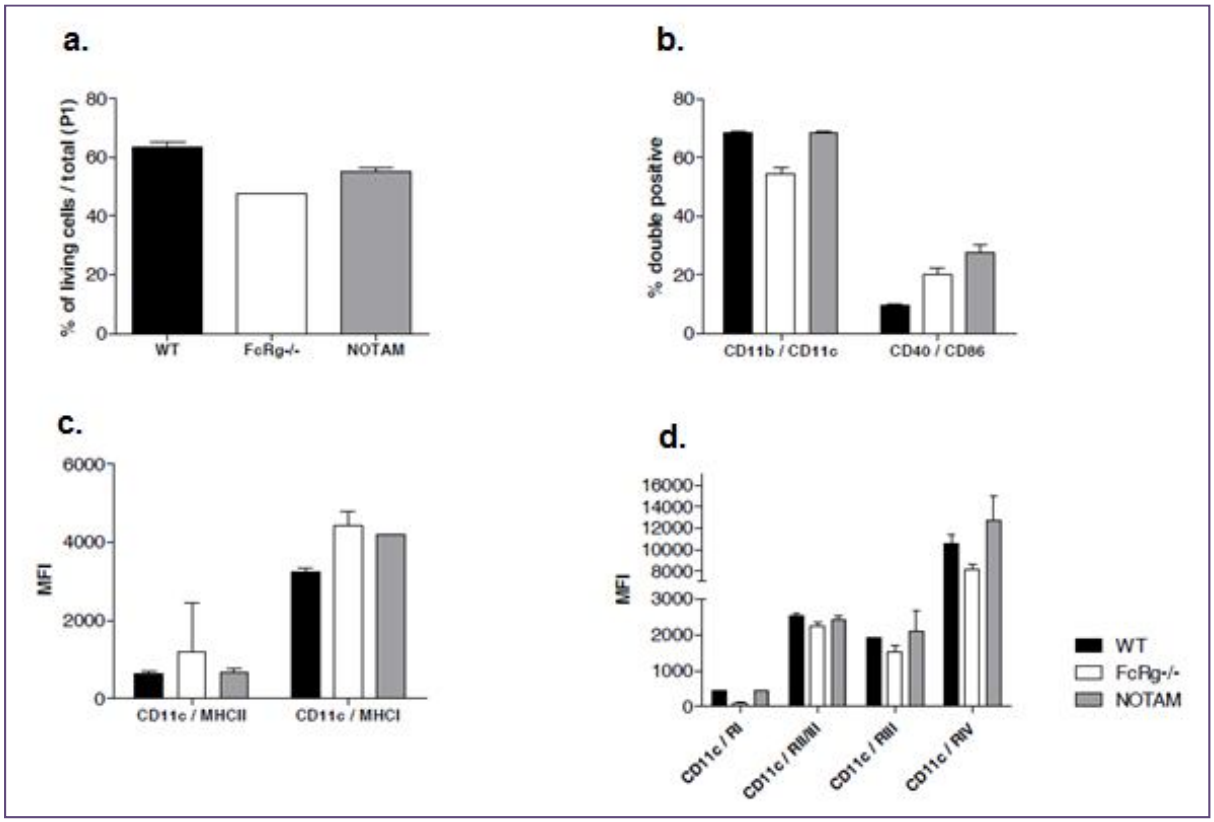

Figure 14: FACS analysis of BMDC from tree types of mice. The results are described in the text. (A) Percentage of living cells derived from WT, FcγR$^{-/-}$ and NOTAM mice. (B) Percentage of activated cells shown as CD40+/CD86+ cells of the three types of mice. (C) Fluorescence corresponding to MHCI or MHCII expression in DC of the three types of mice. (D) Expression of FcγRI-FcγRIV on the surface of DC from the three types of mice.

Immunecomplex binding assay

Figure 15 shows the results of the FACS analysis of the immune complex binding assay. Filled dots are samples without TB, thus showing the total of binding and uptake of immune complexes. The open dots are with TB, representing only the uptake. The difference between a filled and an open dot is the amount of bound IC. Two stimuli, CpG (TLR9 ligand) and PMA (a general PKC ativator) are tested for their effect on immune complex binding or uptake. WT DC show normal binding and uptake of ICs (Figure 15a and 15b). CpG or PMA do not have significant effects. The binding and uptake of ICs in FcRγ$^{-/-}$ mice DC is, as expected, almost zero (Figure 15c and 15d). IC binding by NOTAM DC, however, is unexpected (Figure 15e and 15f). It was shown before that NOTAM mice can bind and take up ICs, but to a lower extent compared to WT DC. In this experiment NOTAM DC show a very low binding compared to WT DC. Uptake of ICs was not increased compared to FcRγ$^{-/-}$ DC.

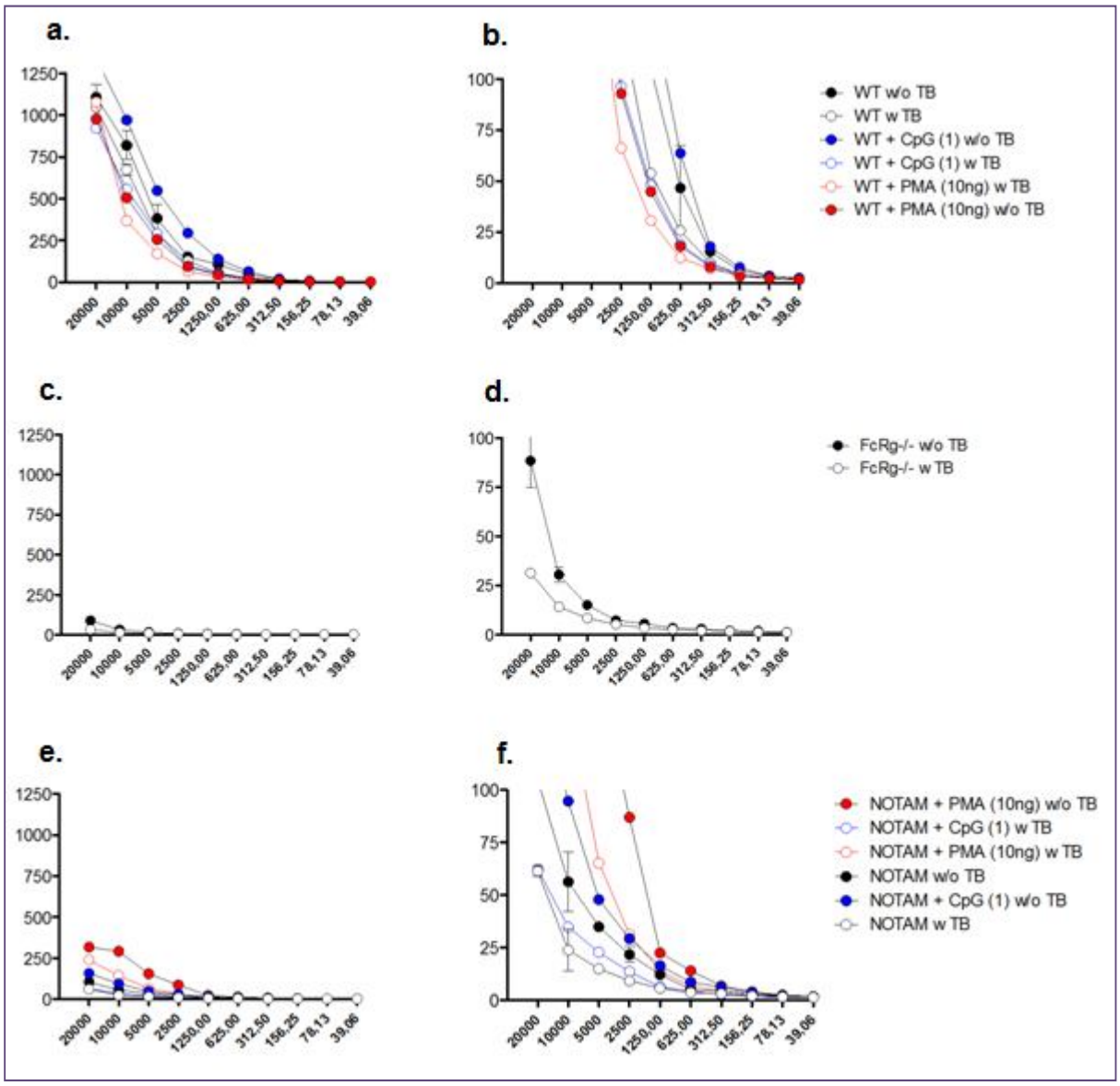

Figure 15. Results of the immune complex binding assay. The graphs are described in the text. Graphs in the right panel are a close up from the graphs in the left panel. (A,B) WT mice, (C,D) FcγR$^{-/-}$ mice and (E,F) NOTAM mice.

Degradation assay

To see whether OVA-IC is degraded after uptake in DC, which is necessary for antigen presentation, a degradation assay has been performed. In this assay OVA-Alexa488-ICs were incubated with DC, excess IC was washed away and the cells were lysed. The lysate was analyzed on SDS-PAGE elecrophoresis and OVA degaradation products were detected by scanning the gels for fluorescent signals. Figure 16 shows the results of the degradation assay. The thickest lane is intact OVA. In WT mice a second lane right under intact OVA becomes clear over time, this represents degraded OVA. After 1 or 2 hrs degradation products of OVA are seen in WT cells. The FcRγ$^{-/-}$ DC show no degradation, except at 24 hrs. This is probably due to non-specific uptake of OVA through mannose receptors. Samples from NOTAM DC also lack the band of degraded OVA. However, the band of intact OVA is thicker than in FcRγ$^{-/-}$ mice, which indicates that ICs are bound to the cell via FcγRs in NOTAM DC. As a control, OVA without an antibody was added to cells of the three types of mice. Under these conditions uptake of OVA by the DC is inefficient and only very little OVA is detected in these samples.

In Figure 17 the results of another degradation assay are shown. In this experiment ProtK treatment is used in some samples to remove OVA-ICs bound from the cells, with the aim to only analyze OVA that is taken up by the DC. The samples without ProtK are in accordance with the results of the degradation assay of Figure 5. ProtK treatment was performed on +4°C to keep the cells and only extracellular proteins are now degraded. ProtK activity was not stopped at the end of the assay, therefore most likely at the time of the lysis of the cells some ProtK activity was still present. As a result, internalized OVA is probably also degraded. The extra bands in the ProtK conditions could therefore be explained by partial degradation product of cell-bound OVA-IC or degradation of internalized OVA. Thus, unfortunately the results are inconclusive.

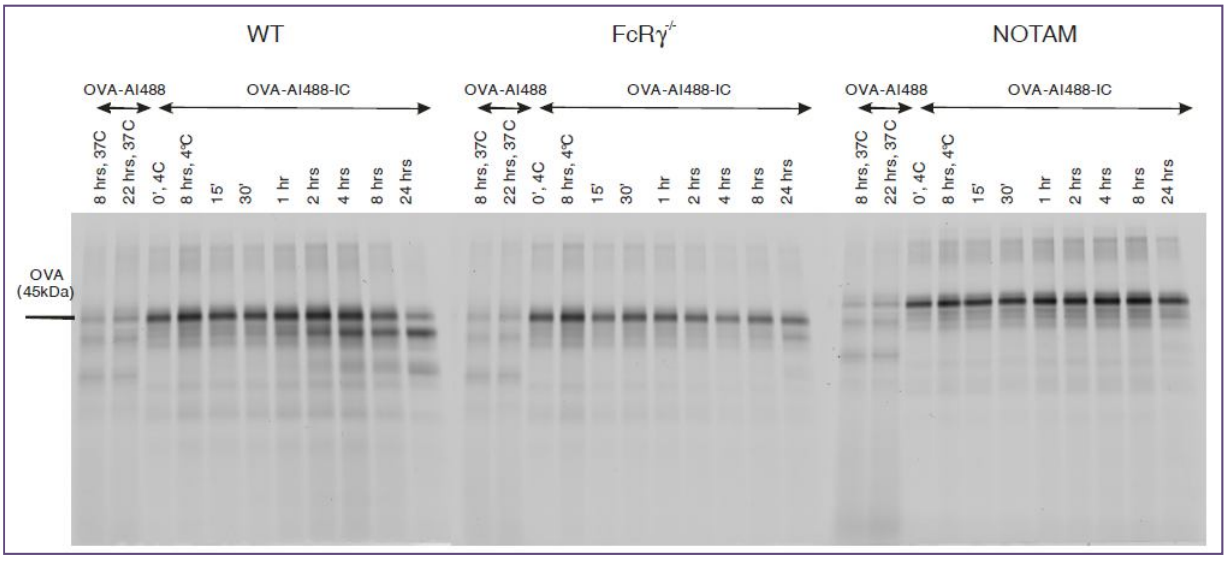

Figure 16.Western Blot results of degradation assay. The thick lane represents intact OVA in WT cells, FcγR$^{-/-}$ cells and NOTAM cells. The lane right under the OVA lane is represents degraded OVA. The results are described in the text.

Discussion

The phenotyping results were as expected. FcγRI, FcγRIII and FcγRIV expression in NOTAM DC was similar to the expression in WT mice, and absent in FcγR$^{-/-}$ mice which is due to the lack of the γ-chain. The higher expression of CD40/CD86 and MHC-I and MHC-II in NOTAM and FcγR$^{-/-}$ mice has been seen before. The exact cause of this 'more activated' state of DC is not clear. In the past however, this has not been a problem in the assays. These results show that the cultured DC can be used in the assays. Binding and uptake of OVA-IC was normal in WT DC and almost absent in FcγR$^{-/-}$ DC, as expected. However, binding and uptake in NOTAM DC is also very low, while it was seen previously that these DC are as efficiently as WT DC in binding and uptake of OVA-IC. The phenotyping results were good, so maybe something went wrong during the assay. The experiment should be repeated. Furthermore, addition of CpG has no effect on the binding and uptake of ICs. Addition of PMA increased IC binding by NOTAM DC but decreased IC binding by WT DC.

As expected, degradation was seen in DC of WT mice over time, but not in FcγR$^{-/-}$ DC which lack the expression of the FcγRs necessary for OVA-IC binding and uptake. In NOTAM mice DC also no degradation was observed. Whether this is due to reduced uptake or reduced degradation cannot be determined because this assay does not discriminate between extracellular and intracellular undegraded OVA. A second degradation assay using also ProtK treatment has been performed to separate these two conditions. ProtK cleaves off OVA-IC bound to the surface of the DC, so when OVA-IC is still present in the NOTAM samples after ProtK treatment this means that OVA-IC has been internalized. When degradation products are seen too, this means that the ITAM signaling is not necessary for the internalization and degradation of OVA-IC. Unfortunately, no conclusions can be drawn from this assay. Degradation products were visible in FcγR$^{-/-}$ samples treated with ProtK, while no degradation was seen in these samples when not treated with ProtK. Probably ProtK activity was not stopped during the assay. After lysis of the cells, ProtK might have been able to cleave internalized OVA-IC, resulting in the presence of degradation products on the gel. Consequently, this assay has to be repeated.

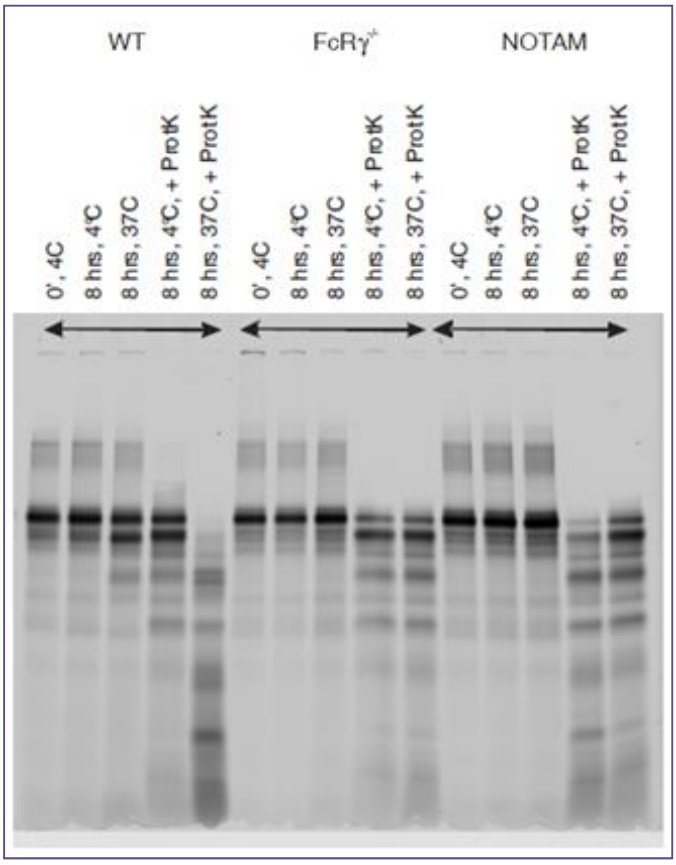

Figure 17. Western Blot results of ProtK degradation assay. The figure is further described in the text.

Appendix V
Confocal microscopy

Introduction

In order to activate T cells, peptides from antigens need to be generated after phagocytosis and presented on MHCII molecules on the cell surface of DC. Antibodies can enhance the process of antigen presentation by interacting with the antigen and FcγR expressed on DC. As mentioned before, some FcγR contain an ITAM motif in the associated γ-chain which is necessary to generate stimulatory intracellular signals. However, the exact role of ITAM in antigen presentation is unknown. To study this, we use WT, FcRγ $^{-/-}$, and NOTAM mice. To test whether immune complexes made of OVA and IgG (OVA-IC) are internalized in DC and presented via MHC-II, confocal microscopy will be used. With this technique it is possible to demonstrate the colocalization of OVA-IC and MHC-II, which indicate the presentation of OVA by DC. Here, we show the results of confocal microscopy from WT mice. The purpose of this experiment was to test different Abs used for fluorescent labeling of proteins, to identify the best combination of Abs with minimal nonspecific binding.

Materials & Methods

DC were isolated from the bone marrow of WT, FcRγ $^{-/-}$, and NOTAM mice and incubated with OVA-IgG immune complexes (OVA-IC). One drop of cell suspension was placed on a cover slip and incubated on ice for 45 minutes, allowing the cells to attach to the glass. Next, cells were fixed with paraformaldehyde and incubated with different Abs to fluorescently label endosomes. An anti-Transferrin receptor and anti-LAMP-1 Ab were tested. OVA-IC was already fluorescently labeled by attaching Alexa594 to OVA. MHC-II molecules were labeled with Alexa647. To label the nuclei DAPI was used.

Results

Figure 18 shows two DC of WT mice incubated with an anti-LAMP-1 Ab. The LAMP-1 signal is strong and should correspond with the location of late endosomes. MHC-II molecules seem to be colocalized with this compartment, as the LAMP-1 and MHC-II signals overlap. The signal of OVA-IC overlaps with MHC-II, suggesting that there is an interaction between these two molecules. Staining of nuclei with DAPI is very weak. Figure 19 shows strong staining of nuclei. These cells were incubated with an anti-Transferrin receptor Ab, which signal is much weaker than that of anti-LAMP-1 Ab in Figure 18. However, it is still possible to see the staining of early endosomes by this Ab. Two or three cells are positive for MHCII staining, the other cells are probably not DC. OVA-IC in the MHC-II positive cells seems to be colocalized with the MHC molecules. OVA-IC staining is also seen in MHC-II negative cells.

Discussion

The DAPI signal in Figure 18 is very weak in comparison with the signal in Figure 19. DAPI signal weakens in time and probably the sample of Figure 18 was an older one. Colocalization of OVA-IC and MHCII is seen, probably showing antigen presentation in DC. OVA-IC might be also internalized by other cells than DC, because OVA-IC signal is also present in MHC-II negative cells. In order to distinguish DC from other cell types, it is important to always label for MHCII. To locate the endosomes, the anti-LAMP-1 Ab is probably more useful than the anti-Transferrin Ab. The signal of

anti-LAMP-1 Ab is stronger and less scattered throughout the cell than the anti-Transferrin Ab signal. The next step would be to test the anti-LAMP-1 Ab in DC of FcRγ $^{-/-}$ and NOTAM mice.

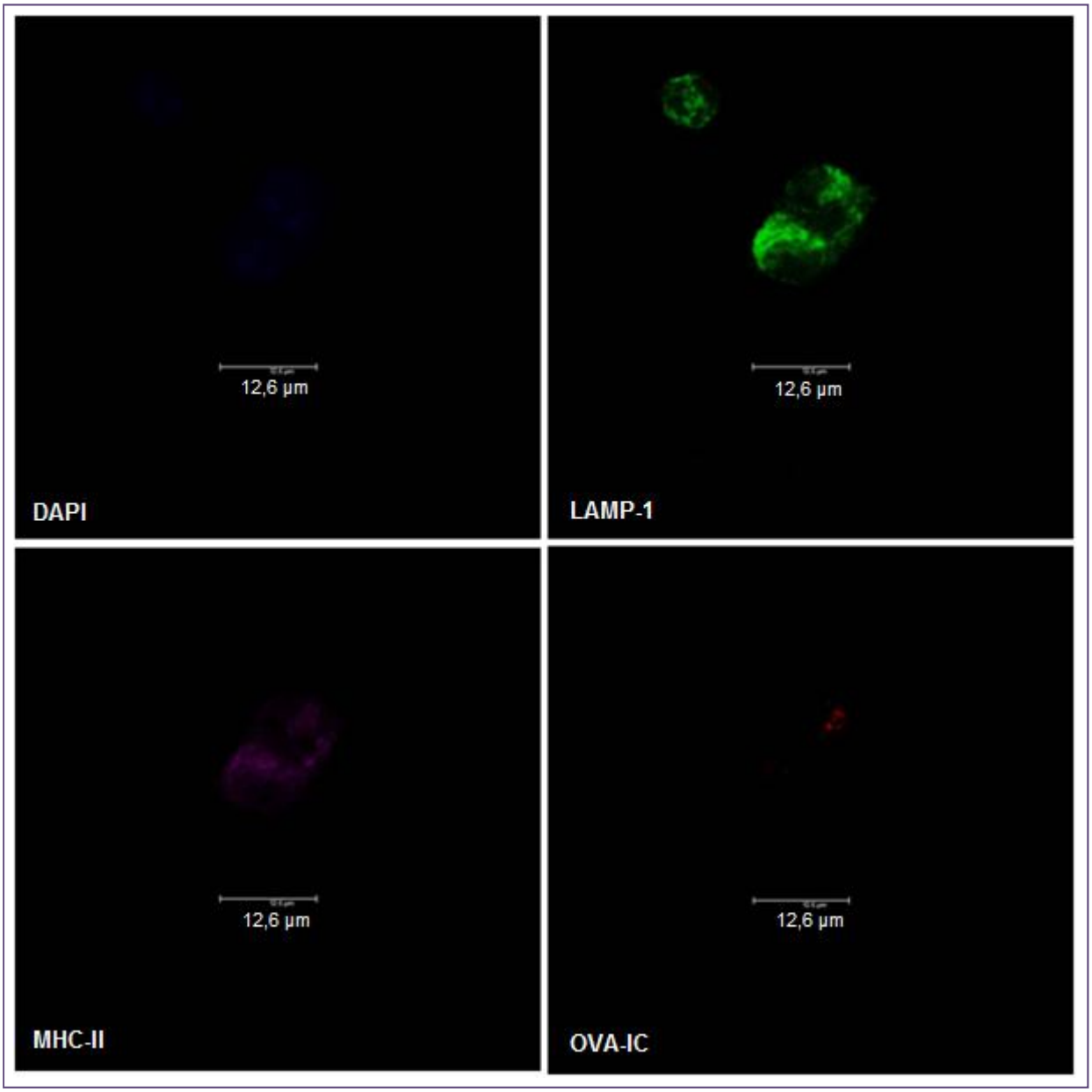

Figure 18. Confocal microscopy results. Four different staining of DCs of WT mice: DAPI, LAMP-1, MHC-II and OVA-IC. LAMP-1 staining corresponds with late endosomes. OVA-IC staining shows where immune complexes of Cetuximab and Alexa594 labeled OVA are located. Colocalization is done with Alexa647 labeled MHC-II molecules.

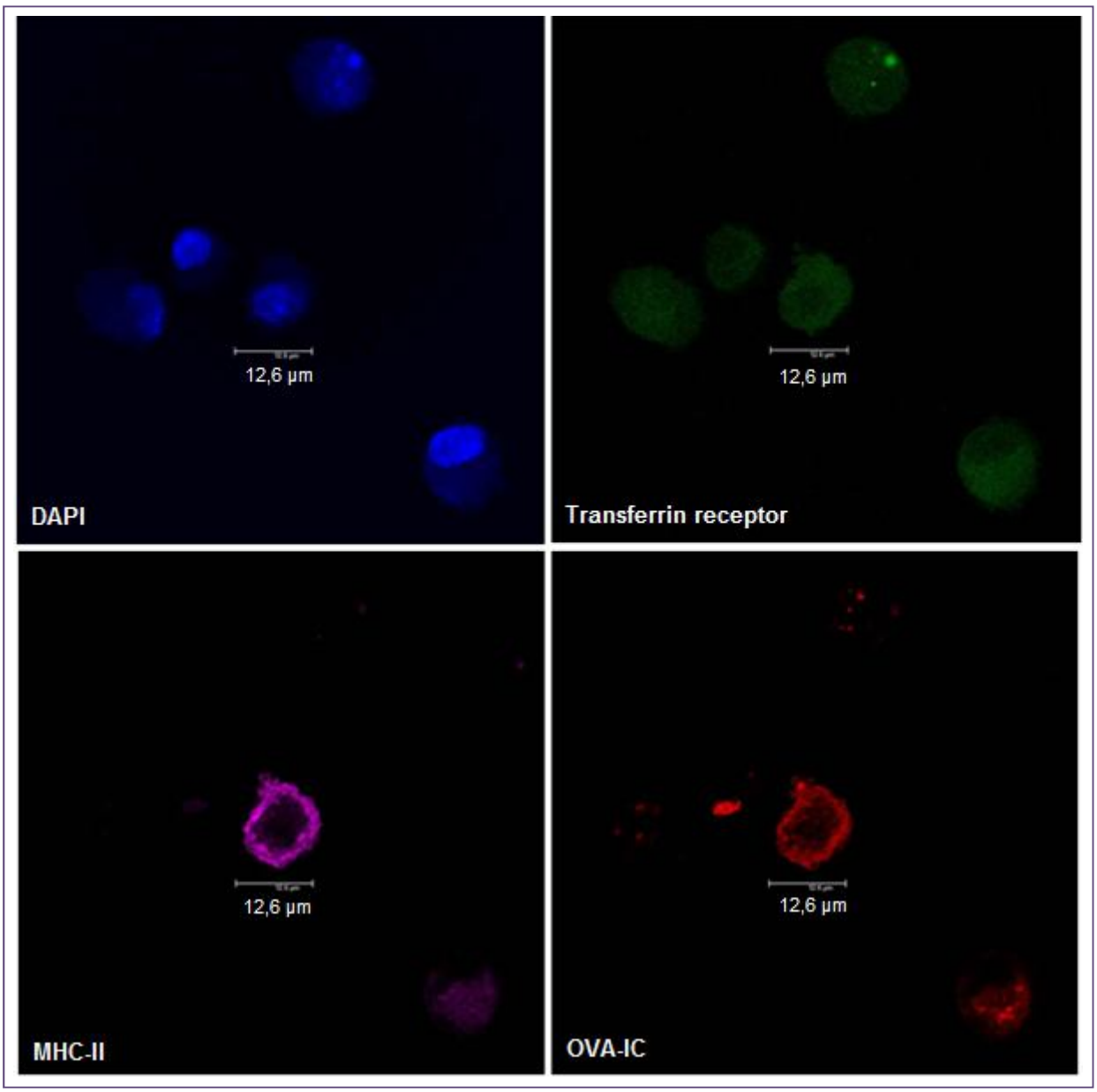

Figure 19. Confocal microscopy results with Transferrin receptor. Four different staining of DCs of WT mice: DAPI, Transferrin receptor, MHC-II and OVA-IC. Transferrin receptor staining corresponds with early endosomes. As in Figure 18, OVA-IC staining overlaps with the MHC-II staining.

Appendix VI
Protocols of experiments described in Appendix IV

Protocol 1: Preparation bone marrow DC/macrophages

Day 0

(GDL)

- Sacrifice the mouse by cervical dislocation (6-16 wks old B6 mouse; preferentially 8-12 wks)
- Cut out femur and tibias and remove muscles with a tissue and tweezers
- Place bones in PBS to prevent dry-out

(Lab)

- Cut the ends of the bones as far towards the ends as possible
- Flush the bone marrow into a 50 ml tube with PBS using a short needle (fill till 50 ml for washing)
- Suck isolated DM through an 18G needle to disaggregate larger BM pieces
- Spin down suspension (1200 rpm for 5 min)
- Lyse erythrocytes by adding 5 ml lysisbuffer pH 7.4 for 2 min at 4 degrees (on ice)
- Add up with PBS to 25 ml
- Put suspension through a cell strainer (100µm)
- Spin down suspension (1200 rpm for 5 min)
- (Re-suspend in PBS and filter to remove large BM pieces; spin down again)
- Re-suspend in 2 ml PBS (amount depends on size of pellet)
- Count cells
- Use Falcon 6-wells plates (not nunc plates!) and filtered medium to culture DC/macrophages (to prevent DC activation during culturing)
- Add GM-CSF (1µl stock (-20C, small lab) to 100ml) to medium to drive differentiation towards DC
    - Refresh the GM-CSF in your medium stock each week (otherwise it is too old to function)
- Plate out $3x10^6$ cells per well (a portion will adhere and differentiate towards macrophages, the rest will remain in suspension and differentiate towards DC)

Day 1 (or day 2)

- Carefully aspirate 2,5 ml medium (progenitors will stay at the bottom)
- Add 3 ml fresh medium

Day 4 (or day 5)

- Refresh medium as on day 1

Day 7

- Suspension cells (DC) can be harvested
- Check phenotype with $CD11b^+$ $CD11c^+$ double staining
- (Adherent cells (macrophages) can be harvested with 50mM EDTA for 10min incubation at 37C and vigorous pipeting)

Protocol 2: Immunecomplex binding assay

- Distribute 10e5 cell/well (V-bottom plate) in 50ul full medium
- Use apart plate for 4C and 37C
- Place it on ice for 20min, cool plate with IC dilution too
- Pipette IC to cells at the indicated timepoints in 50ul medium
- At the end wash with cold FACS buffer in cold centrifuge
- Stain with Abs on ice for 1hrs (CD11b and CD11c (and) a-r-FITC)
- Resuspend in 100ul FACS
- Measure on FACS Calibur

Protocol 3: Degradation assay

- Distibute 1x10e5 DC / Eppendorf
- Spin 3 min at 0,2 rcf, resuspend in 25ul DC medium
- Add OVA or OVA-IC in 25ul normal medium (2x dilution)
- Incubate for 0, 15 or 30 min or 1, 2, 4, 8 or 24 hrs at 4 or 37˚C

Stripping by ProtK treatment

- Repeat previous four steps
- Spin and resuspend cells on ice in ProtK buffer (or PBS but no detergent)
- Add ProtK (0.5mg/ml) for 30 min on ice
- Add 1 volume of 2mM PMSF in buffer to block the ProtK. Final concentration 1mM
- (Prepare 100mM stock in EtOH. Add to buffer when constant vortexing to final concentration 2mM. Just prior to use, even now some precipitation can occur.)

- Wash 2x with 800ul cold PBS, spin in Eppendorf cup at 0,3 rcf
- Pellet lysed in 20ul reducing Sample Buffer (2x) (cc 2x10e6 c/100ul)
- Cook for 5 min at 100˚C
- Store samples at -20˚C

- Run on 15% PAGE, 15 combs, 1.5mm, 20ul sample / slot
- Scan gel on Typhoon Variable Mode Imager at an excitation wavelength of 488 nm

Protocol 4: Western Blot Protocol

Making of gel

- Make a 15% running gel. For composition use table *(Add APS and TEMED together at the end!)*

15% running gel (eg. γ-chain)	**Stacking gel** (4 ml)
4.6 ml H2O	2,7 ml H2O
10 ml Acrylamide mix	670 µl Acrylamide mix
5 ml 1,5M Tris	500 µl **1M Tris**
200 µl 10% SDS	40 µl 10% SDS
200 µl 10% APS	*40 µl 10% APS*
16 µl TEMED	*8 µl TEMED*

- Put some water on the gel, leave some gel in Falcon to see when polymerization complete
- When the gel polymerized, pour water off (use tissue if needed)
- Add staking gel
- When the entire gel is polymerized, place it with wet tissue in foil in the fridge and can be kept for couple of days

SDS PAGE

- Cook samples for 5 min in heatblock at 100˚C
- Place gel in holder and fill up with 1x Electrophorese buffer
- Load wells using Biorad pipette tips
- If the middle compartment is leaking, fill up the outer compartment totally with buffer
- Run for 1-1.5 hrs at 100-120 V

Blotting

- Wash nitrocullulose blot with
    - MethOH (10 sec)
    - Water (2 min)
    - Blotbuffer (5 min)
- Soak sponges (2x) and Watman paper (3x) in blot buffer
- Prepare for blotting while the gel is running
    - Black side down
    - Sponge
    - 3 pieces of Watman paper
    - Gel
    - Nitrocellulose blot
    - 3 pieces of Watman paper
    - Sponge
- Loose gel with green piece of plastic, get rid of stacking gel, apply gel carefully to Watman paper
- Place gel in blotbuffer before this (5 min)
- Place cooling elements in holder
- Run 1-1,5 hr at 400 mA (or o/n at 25mA in cold room in case of big proteins)

Block / Wash / Staining

- Wash 3x with PBST (PBS + Tween20)
- Wash 2x with PBS
- Wash with ECL
- Block with PBSP (PBS + 5% Protifar) for 1 hour at RT
- Incubate with the 1st antibody (rabbit anti-human-γ-chain (Upstate) 1:1000 in 90% PBST, 10% PBSP o/n)
- Incubate with the 2nd antibody (goat anti-rabbit-HRP (Pierce, 25D))

Blot stripping

- Place blot in 50ml Falcon with Stripbuffer for 30min-1h in 55˚C water bath
- Wash few times short with water
- Wash with a lot of TBS-T (or the washing buffer you use)
- After stripping you need to block the blot again and develop as in the protocol

Buffer compositions

Tris/HCl 1.5M PH 8.9 – 9.1g Tris in 40ml Tridest, set pH at 8.9, fill up to 50ml, filter 0.45µm

Tris/HCl 0.5M PH 6.9 – 3.03g Tris in 40ml Tridest, set pH at 6.9, fill up to 50ml, filter 0.45µm

10x Electrophorese buffer

30.3g Tris
144g Glycine
10g SDS
Fill up to 1l with tridest
pH 8.4

5x Blot buffer

56.5g Glycine
12.1g Tris
Fill up to 1l with bidest water
pH 8.4

1x Blot buffer

600ml bidest water
200ml 5x blotbuffer
200ml Methanol
Keep this order because of precipitation
Keep blotbuffer in fridge and reuse it 3x

4x Sample buffer

25mL Milli-Q Water
25mL 1M Tris, pH 6.8
40mL Glycerol
8g SDS
8mL ß-Mercapto-ethanol (if reducing)
400mg Bromophenol Blue
Once SDS and BB are dissolved, filter with a 0.2µm syringe filter.
Store aliquots in -20ºC

Strip buffer

36.5ml water
3.1ml 1M Tris pH 6.8
10ml 10% SDS
0.39ml ß-ME