MHC II and the endocytic pathway; Regulation by Invariant Chain

Thesis for the degree of Philosophiae Doctor

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Abbreviations

AP	Adaptor protein
APC	Antigen presenting cell
ARF	ADP ribosylation factor
BcR	B cell receptor
BMDC	Bone-marrow derived dendritic cell
BSA	Bovine serum albumin
CCD	Charge-coupled device
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CIITA	MHC class II trans-activator
CIIV	MHC class II vesicle
CLIP	Class II associated Invariant chain peptide
COPII	Coat protein complex II
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EEA1	Early endosomal antigen 1
EGFP	Enhanced green fluorescent protein
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
FcRn	Neonatal Fc receptor
FRET	Fluorescence resonance energy transfe
FYVE	PtdIns(3)P binding motif named after its 4 founding members: Fab1, YOTB, Vac1 and EEA1
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HLA	Human leukocyte antigen
HOPS	Homotypic vacuole fusion and protein sorting
Hrs	Hepatocyte-growth-factor-regulated tyrosine-kinase substrate
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain, also called CD74
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAMP	Lysosome associated membrane protein
LC	Langerhans cell
LIMP	Lysosome integral membrane protein
LPS	Lipopolysaccharide
M6PR	Mannose 6-phosphate receptor
MARCH-1	Membrane-associated RING-CH 1
MDCK	Madin Darby canine kidney
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIIC	MHC class II compartment
moDC	Monocyte derived dendritic cell
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MVB	Multi-vesicular body
Mθ	Macrophage
NF-κB	Nuclear factor-κB
NK	Natural killer
NOS2	Nitric-oxide Synthase 2
NOX2	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
PAGFP	Photo-activable green fluorescent protein
pDC	Plasmacytoid dendritic cell
PI-K	Phosphatidylinositol kinase
PKC	Protein kinase C
PMT	Photo-multiplier tube
PRR	Pattern recognition receptor
PtdIns	Phosphatidylinositol
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
SNARE	Soluble N-ethylmalemide-sensitive factor (NSF) attachment protein receptors
SR	Scavenger receptor
TAP	Transporter associated with antigen processing
TcR	T cell receptor
TEC	Thymic epithelial cell
TGN	Trans-golgi network
TH	T helper cell
TLR	Toll-like receptor
TM	Trans-membrane
TNF	Tumour necrosis factor
VAMP	Vesicle associated membrane protein

INTRODUCTION

The immune system serves to protect us against infectious agents (virus, bacteria, fungi and parasites) which manage to penetrate the epithelial surfaces of the body. It is generally divided into two parts, where the innate immune system recognizes foreign entities based on features common to groups of pathogens with invariant pattern recognition receptors (PRRs), and the adaptive immune system targets individual pathogens through the random generation and specific selection of antigen targeting modules (T cell receptors and antibodies). The adaptive immune response can be divided further into cell-mediated- and humoral immunity, characterized by activation and proliferation of T cells, and production of antibodies by B cells, respectively. Both branches of the adaptive immune system require the licensing of pathogen specific T cell clones by antigen presenting cells (APCs). APCs presenting pieces of antigen (peptides) on major histocompatibility complex (MHC) class I molecules activate $CD8^+$ T cells and induce their differentiation into cytotoxic T cells which eliminate infected cells presenting the same antigen on their MHC I. APCs presenting antigenic fragments on MHC class II molecules activate helper ($CD4^+$) T cells, which in turn activate B cells presenting the same antigen on their own MHC II. Activated B cells differentiate into plasma cells that produce antibodies which are released into the extracellular fluids where they attach to their antigens, neutralizing them and marking them for destruction by phagocytes and the complement system. Activated $CD4^+$ T cells can also contribute to cell-mediated immunity by stimulating APCs to become more efficient stimulators of $CD8^+$ T cells, and on innate immunity by delivering activating signals to phagocytic cells. The presentation of antigenic peptides on MHC II is thereby important for both adaptive and innate immunity against infectious agents. MHC II assembly in the endoplasmic reticulum (ER) and trafficking to endosomes for peptide loading is guided by a specialized chaperone termed the invariant chain (Ii). Ii self-associates into a trimer in the ER, this provides a scaffold for the assembly of three MHC II heterodimers and blocks their peptide binding grooves to avoid premature binding of endogenous peptides in the ER and Golgi apparatus. Ii then transports MHC II to endocytic compartments where they encounter internalized pathogens and modulates the endocytic environment to facilitate the generation of diverse arrays of pathogenic peptides. Thus, in the absence of Ii the assembly of MHC II is impaired and the repertoire of antigenic peptides displayed is significantly compromised. Ii is therefore crucial to achieve the efficient antigen presentation on MHC II and consequently for APC function in innate and adaptive immune responses. The aim of this thesis was to investigate the molecular mechanisms involved in the loading of diverse repertoires of antigenic fragments on MHC II with a special focus on the roles played by the versatile Ii molecule.

ANTIGEN PRESENTING CELLS

Whereas MHC I is ubiquitously expressed on all nucleated cells, the expression of MHC II is generally restricted to a limited set of cells, which include B lymphocytes, cells of the monocyte-macrophage lineage (Mθs) and dendritic cells (DCs). These three constitute the so-called *professional* antigen presenting cells (APCs), and are more or less specialized to acquire, process and present antigens

(Trombetta and Mellman, 2005). In humans (but not in mice) activated T cells can also express MHC II, and thymic epithelial cells (TECs), presenting endogenous peptides on MHC II, play an vital role in the development of CD4+ T cells. Through positive and negative selection TECs direct the development of the T cell receptor (TcR) repertoire in the thymus (Derbinski and Kyewski, 2010; Starr et al., 2003). Finally MHC II expression can be induced in a number of other cell types by interferon (IFN)-γ (Harton and Ting, 2000; Pattenden et al., 2002; Pober et al., 1983).

B LYMPHOCYTES

The principal function of B cells is to make antibodies to identify and neutralize foreign objects. As such, they primarily present fragments of antigens recognized and internalized via their clonally specific, cell surface expressed, immunoglobulin, the B cell receptors (BcR). Although antigen recognition and uptake via the BcR can in some cases enable B cells to activate naïve T cells (Rodriguez-Pinto, 2005; Rodriguez-Pinto and Moreno, 2005), the relative low frequency of naïve B cell clones with a BcR specific for any particular antigen and their absence from mucosa and skin make them inadequate as antigen presenters to initiate adaptive immune responses. Nevertheless, the presentation of antigen by B cells to activated, antigen specific, helper T cells is essential for their development into antibody producing plasma cells (Parker, 1993), and the class of antibody produced depends on the type of helper T cell involved and the cytokines released (King et al., 2008).

MACROPHAGES

Mθs are a diverse population of phagocytes which in the steady state scavenge cellular debris and facilitate tissue remodeling (Geissmann et al., 2010b; Mosser and Edwards, 2008). During infection they are particularly adept at the destruction of pathogens due to the expression of a broad range of phagocytic receptors, and a particularly proteolytic endo-lysosomal system (Aderem and Underhill, 1999; Gordon, 2002). In response to interferon (IFN) γ and PRR stimulation, the processing of intracellular pathogens is further enhanced as part of the microbial response (MacMicking et al., 2003; Santic et al., 2005; Xu et al., 2007). Conversely this feature which makes them so adept at destroying intracellular microbes eliminates many T cell epitopes and thereby negatively impacts on their capacity to present diverse repertoires of antigenic fragments on MHC II. Alternative activation with interleukin (IL)-4 and/or IL-13 results in Mθs with a lower production of toxic oxygen and nitrogen radicals, and decreased ability to kill intracellular bacteria, but these Mθs express little or no co-stimulatory molecules and fail to stimulate naïve T cells (Edwards et al., 2006). In keeping with their primary function in clearing the interstitial environment of extraneous cellular material, Mθs are mostly tissue resident and do not efficiently migrate to lymphoid organs where the majority of naïve T lymphocytes are found. Consequently Mθs fail to present their acquired antigen to wide repertoires of T cell clones, and are therefore not considered sufficient to maintain adaptive immunity.

DENDRITIC CELLS

The by far most potent stimulators of naïve T cells are the DCs. These cells can be found throughout the various tissues of the body where they act as sentinels to discover and alert the immune system to the presence of foreign entities (Figure 1). DCs continuously sample their environment via diverse endocytic mechanisms and they express a wide range of PRRs which upon ligation initiates a

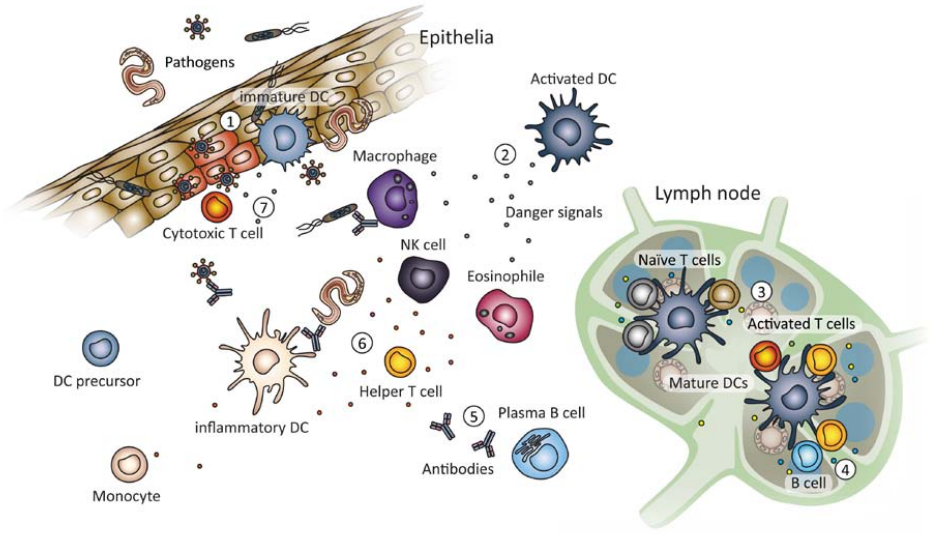

FIGURE 1 | FUNCTION OF DENDRITIC CELLS ACCORDING TO THE LANGERHANS CELL PARADIGME. DCs act as sentinels in tissues by sensing the local environment (1) and respond to pathogens (e.g. viruses and bacteria) or inflammatory stimuli (e.g. TNF-α or IL-1β) by differentiating into mature DCs. Mature DCs act locally by secreting chemokines and cytokines which recruit and activate innate immune cells (e.g. NK cells, MΘs and eosinophiles) (2), and they migrate to secondary lymphoid organs where they activate naïve antigen-specific T lymphocytes (3). Activated helper T cells can direct B cells to differentiate into plasma cells (4). Plasma B cells secrete antibodies which attach to antigens, neutralizing them and marking them for destruction by phagocytes and the complement system (5). T helper cells also produce cytokines locally for the recruitment and activation of innate immune cells (6). Activated cytotoxic T cells target infected cells directly by inducing their apoptosis or releasing lytic proteins (perforin, granulysins and granzymes) (7). Figure based on (Banchereau et al., 2000).

complex differentiation program termed "maturation". Mature DCs are characterized functionally by their ability to prime immune responses and phenotypically by the expression of high cell surface levels of MHC II and costimulatory molecules (discussed in (Reis e Sousa, 2006)). This is due to diverse intracellular events which influences: i) the uptake and processing of antigens, ii) the production, peptide loading and cell surface stability of MHC II, iii) the expression of cytokine receptors and adhesion molecules involved in migration, iv) the release of cytokines for recruitment and activation of innate immune cells and v) the expression of co-stimulatory molecules and secretion of cytokines for the activation and polarization of naïve T cells (Banchereau et al., 2000).

DENDRITIC CELLS ORCHESTRATE IMMUNE RESPONSES

Depending on the activating ligand, the extracellular milieu at the time and place of activation and the type of DC which is activated, mature DCs direct the polarization of antigen specific naïve T cells to facilitate the appropriate responses (Dorhoi and Kaufmann, 2009). Thus, influenza virus dsRNA, binding TLR3, induces the secretion of IL-12 by human monocyte derived (mo-) DCs, T_H1 development, and immunity against intracellular pathogens (Cella et al., 1999). Development of T_H2

cells mediating host defense against parasitic infections can be induced in mice by bacterial peptidoglycan derived muramyl dipeptide binding the cytosolic PRR Nod2 in mouse bone marrow derived (BM-) DCs (Magalhaes et al., 2008), but also by the fungal cell wall component Zymosan, which triggers the release of IL-10 by mouse splenic $CD8\alpha^-$ DCs (Manickasingham et al., 2003). Conversely, Zymosan binding the beta-glucan receptor Dectin 1 and Toll-like receptor (TLR) 2 on human moDCs triggers IL-23 secretion and the development of T_H17 T helper cells crucial for defense against fungi and extracellular bacteria (Gerosa et al., 2008; McGeachy et al., 2009; Milner et al., 2008). In the absence of activating stimuli, or in the presence of immunosuppressive cytokines, DCs can also induce the development of regulatory T cells, induce T cell anergy or elimination (Pulendran et al., 2010; Steinman et al., 2003; Yamazaki and Steinman, 2009) (Figure 2).

In addition to their potent effect on naïve T cells, DCs also influences the activity of other immune cells by releasing signals which recruit and activate innate immune cells. During inflammation, activated DCs release chemokines such as CCL4 (MIP-1β), CCL5 (RANTES), CX3CL1, CXCL8 (IL-8) and CXCL10 (IP-10) which recruits natural killer (NK) cells to sites of inflammation (Megjugorac et al., 2004; Papadopoulos et al., 1999). Type 1 IFNs released by activated plasmacytoid DCs preferentially augments protective NK cell cytotoxicity (Gerosa et al., 2005), whereas activated myeloid DCs secrete IL-12 and IL-18 which induces IFNγ production by NK cells (Andoniou et al., 2005; Ferlazzo et al., 2004). IFNγ is an important inflammatory cytokine, which is also

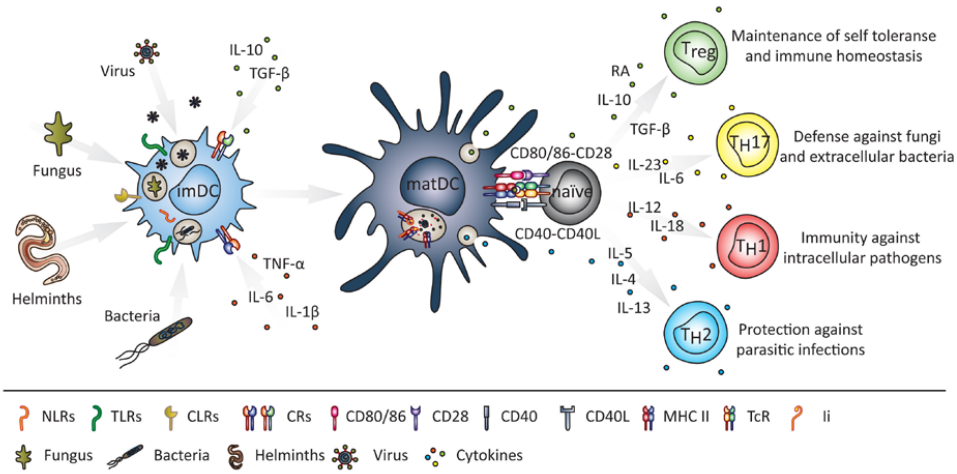

FIGURE 2 | DENDRITIC CELLS INTERPRETE ENVIRONMENTAL CUES AND DIRECT T CELL RESPONSES. DCs can sense pathogens directly through various invariant pattern recognition receptors but also indirectly, through factors secreted by other cells in the micro-environment. Stimulation of distinct signaling pathways mediates the production of different cytokines and factors that control TH polarization. Naïve CD4+ T cells recognizing a specific peptide-MHC II complex and activated through co-stimulatory ligands, develop into different lineages depending on the combination and level of cytokines present. Thus, IL-12 and IL-18 induce T_H1 responses and IL-4 together with IL-5 and IL-13 favor T_H2 differentiation. TGF-β promotes either T_{reg} or T_H17 polarization, depending on its abundance and on other cytokines. Based on (Dorhoi and Kaufmann, 2009; Pulendran et al., 2010). NLRs, Nod-like receptors; TLRs, Toll-like receptors; CLRs, C-type lectin receptors; CRs, cytokine receptors; TcR, T cell receptor; MHC II, major histocompatibility complex class II molecules; Ii, invariant chain.

produced by antigen specific T_H1 cells, that has multiple effects on the immune response, including activation of macrophage anti microbial activity, enhanced NK cell cytotoxicity and induction of MHC II and increased MHC I expression in diverse cell types. Importantly, IFNγ also leads to the recruitment of monocytes to infected sites where they can differentiate into Mθs (Gordon and Taylor, 2005), TNFα and iNOS producing inflammatory DCs important for IgA production and clearance of gram positive bacteria (Kang et al., 2008; Serbina et al., 2003; Tezuka et al., 2007), dermal monocyte derived DCs producing IL-12 and T_H1 responses (Leon et al., 2007) or DC-SIGN/CD209a positive DCs that actively home to lymph nodes in a L-selectin and CCR7 dependent manner and efficiently activate both $CD4^+$ and $CD8^+$ T cells (Cheong et al., 2010). Monocytes thereby contribute to the steady state DC network by differentiating into distinct mo-DC types at sites of inflammation.

Heterogeneity in the Dendritic Cell network

The heterogeneity in the DC network is bewildering, and the relationship between DC subtypes, monocytes and Mθs is still debated (Geissmann et al., 2010a). This is due to the variable, often overlapping expression of the various cell surface markers used in their classification, their adaptability to the various tissues they inhabit and their plasticity in response to environmental cues. Also, the differences in DC biology between mice and humans makes extrapolation of findings in mice to the human DC systems complicated. Nevertheless, most DC biologists recognize two main categories of DCs in both species: plasmacytoid DCs (pDCs) and conventional DCs. The pDCs are long lived cells found in circulation which selectively express TLR7 and TLR9 and upon stimulation with viral compounds release large amounts of type I interferons important for anti-viral immunity (Colonna et al., 2004; Liu, 2005). In keeping with their anti-viral function, pDCs preferentially present endogenous viral antigens in a mature state, and maintain MHC II production and turnover in order to achieve this (Villadangos and Young, 2008; Young et al., 2008).

Conventional Dendritic cells. Conventional DCs can be divided into multiple subpopulations based on their expression of surface markers, functional properties and the tissues they inhabit (Heath and Carbone, 2009; Shortman and Naik, 2007; Ueno et al., 2010b; Villadangos and Schnorrer, 2007). Mouse lymphoid organs contain at least three resident DC subsets distinguished by their expression of CD8α and CD4. These cells develop from bone-marrow derived precursors within the lymphoid organs and remain there through a limited number of divisions before they die and are replaced (Liu et al., 2007; Naik et al., 2006). CD8α positive DCs are particularly adept at cross-presenting exogenous antigens on MHC I (Villadangos and Schnorrer, 2007), they are the most efficient phagocytes of dead cells (Iyoda et al., 2002) and the dominant producers of IL-12 (Hochrein et al., 2001; Reis e Sousa et al., 1997). This suggests that $CD8\alpha^+$ DCs play a major role in directing T_H1 development (Maldonado-Lopez et al., 1999) and activation of cytotoxic $CD8^+$ T lymphocytes in response to cell-associated antigens (Shortman and Heath, 2010). In contrast, the CD8α negative (CD4 positive/negative) DCs are more efficient at presenting antigens on MHC II and induction of T_H2 responses (Dudziak et al., 2007; Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Schnorrer et al., 2006). A third subset expressing neither CD8α nor CD4 (double-negative) is generally included in the $CD8\alpha^-$ DC subset, they

express the same PRRs (Luber et al., 2010) and have a similar cytokine profile in response to TLR agonists (Proietto et al., 2004). Lymphoid organ resident DCs acquire soluble antigens carried through the afferent lymph (Sixt et al., 2005), or antigen transported into lymph nodes by migratory DCs (Allan et al., 2006).

MIGRATORY DENDRITIC CELLS. At least three distinct migratory DCs enter the draining lymph nodes from the skin in the steady state, with related subsets present in the lymph nodes draining the gut, liver, kidneys and lungs (Heath and Carbone, 2009). The langerin (a C-type lectin receptor, also called CD207) expressing Langerhans cells (LCs) come from the epidermis (Schuler and Steinman, 1985), where they are capable of self renewal in the steady state, but are replenished by progenitor cells from the blood during inflammation (Merad et al., 2002). LCs were considered the prototypic DCs and are the origin of the so-called Langerhans cell paradigm of DC function presented in figure 1 (Girolomoni et al., 2002; Wilson and Villadangos, 2004). The central role of LCs in immunity to skin antigens became disputed when they were found to be incapable of priming T cell responses to epidermal virus infection (Allan et al., 2003), and were dispensable for triggering hapten-specific T cell effectors by skin immunization (Kissenpfennig et al., 2005). However, other studies show competent cross-presenting capacity and potent induction of CD8+ T cell effector functions (Stoitzner et al., 2006), for reviews see (Kaplan et al., 2008; Romani et al., 2010). Recently a langerin positive DC subset expressing CD103 was identified in the dermis (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007), these DCs conform to the Langerhans cell paradigm, but are vastly superior to LCs at cross-presenting viral antigens to $CD8^+$ T cells (Bedoui et al., 2009; Henri et al., 2010).

The third DC subset present in the skin in the steady state are the classical dermal $CD11b^+$ DCs. $CD11b^+$ DCs efficiently migrate to draining lymph nodes and preferably present antigens in the context of MHC II to $CD4^+$ T cells (Bedoui et al., 2009; Zhao et al., 2003).

IN VITRO DENDRITIC CELLS. Studies on basic DC function in mice in many cases relies on *in vitro* differentiated DCs (Shortman and Naik, 2007), where bone marrow precursors cultured in granulocyte/macrophage colony-stimulating factor (GM-CSF) or FMS-related tyrosine kinase 3 ligand (FLT3L) give rise to DCs which resemble migratory and inflammatory DCs or $CD8^-$, $CD8^+$ and pDCs respectively (Brasel et al., 2000; Inaba et al., 1992; Lutz et al., 1999; Naik et al., 2005).

DENDRITIC CELLS IN MICE AND HUMANS.
Most of the DCs subsets have been identified and characterized in mice due to the obvious ethical and practical difficulties in conducting such research in humans. However, evidence exists that the DC network is maintained also in humans. Human skin contains LCs in the epidermis and two dermal subsets distinguished by the expression of CD1a and CD14. The $CD14^+$ DCs prime $CD4^+$ T cells that direct plasma cell development but are poor at activating CD8+ T cells, whereas the $CD1a^+$ DCs are intermediate at activating CD4 and CD8 positive T cells and LCs preferentially induce T_H2 development and are superior at priming naïve $CD8^+$ T cells (Klechevsky et al., 2008). Both pDCs and myeloid DCs are found in human blood, and human pDCs like their counterparts in mice, release large amounts of type I interferons upon viral exposure (Siegal et al., 1999). Recently, the human counterparts to mouse lymphoid organ resident $CD8\alpha^+$ DCs were identified, and these $CD141^+$ DCs are also capable of

phagocytosing dead cells and cross-presenting cellular and soluble antigens (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). In humans, as in mice, monocytes readily differentiate into DCs in response to appropriate stimuli, and such DCs readily stimulate T cells in a mixed lymphocyte reaction (Sallusto and Lanzavecchia, 1994). This protocol for the rapid generation of large numbers of DCs from monocytes has been thoroughly exploited in research to study basic human DC function, and attempts have also been made to use these moDCs to combat cancer and infectious disease (Palucka et al., 2010). However, how these moDCs compare to their *in vivo* generated counterparts and steady state DC subsets is poorly investigated. In paper II we investigate the maturation induced effects in human moDCs on the molecules involved in presenting antigen to CD4+ T cells.

MHC II AND Ii, STRUCTURE AND FUNCTION

MHC II is encoded by polymorphic genes and expressed as non-covalent heterodimers of two type I (C-terminal in cytosol) transmembrane (TM) polypeptides. The α (35kD) and the ß (28kD) chain differ in size mainly due to difference in N-linked glycosylation (Kaufman et al., 1984). The luminal domains of the MHC II chains have an intrinsic ability to associate (Kjær-Nielsen et al., 1990; Wettstein et al., 1991), but interactions by the TM domains promote the formation of correctly assembled complexes (Cosson and Bonifacino, 1992). The extracellular part of the MHC II molecule forms a groove composed of two α-helices supported by an eight-strand β-pleated sheet. The groove contains pockets into which anchoring peptide side chains

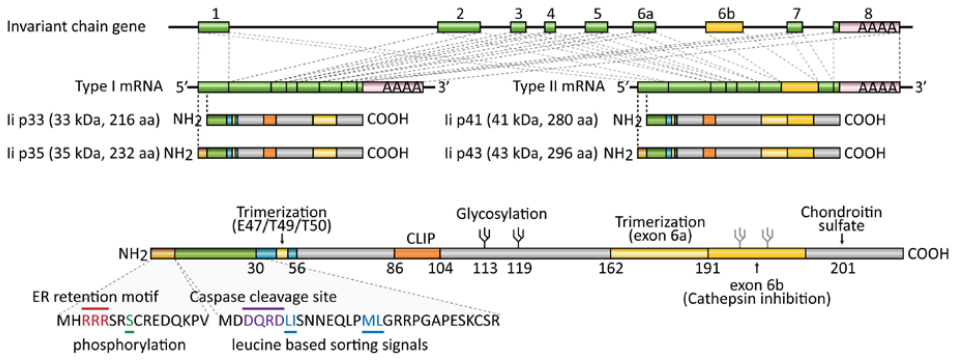

FIGURE 3 | MODULAR STRUCTURE OF THE GENE ENCODING HUMAN INVARIANT CHAIN, ITS MRNAS AND PROTEIN PRODUCTS. The Ii gene yields two mRNA species differing in the 3′ region due to alternative splicing of exon 6. Two alternative in-phase translation initiation codons give rise to four distinct protein isoforms distinguished by a 16 amino acid N-terminal extension (p35 and p43) and a 64 amino acid insertion in the C-terminal region (p41 and p43). The alternative splicing yielding p41 and p43 occurs on approximately 10% of the transcripts and alternative translation from the first translation initiation start codon found in humans gives an estimated 20-30% Ii proteins containing this 16 amino acid extension. The predominant isoform is consequently p33, however, for simplicity p43 is shown. The 16 amino acid N-terminal extension in p35/p43 contains a prototypic ER retention motif (Lotteau et al., 1990) and a phosphorylation site targeted by protein kinase C (Kuwana et al., 1998). All isoforms contain the remainder of the cytoplasmic tail with a putative caspase cleavage site (DQRD) (Huang et al., 2008), two di-leucine based sorting motifs for adaptor protein interactions and a particular charge distribution which is essential for endosomal fusion and delayed maturation. Heterotypic trimerization is mediated by (at least) two domains contained in the transmembrane region and exon 6a, and MHC II association through CLIP (residues 86-104) which binds into the peptide binding groove of MHC II (Freisewinkel et al., 1993; Romagnoli and Germain, 1994; Stumptner and Benaroch, 1997), but also a region C-terminal to CLIP (residues 103-118) (Thayer et al., 1999) and the trans-membrane region (Castellino et al, 2001). All isoforms contain at least two N-linked and two O-linked glycosylations (Machamer and Cresswell, 1984) and a tiny fraction may acquire a chondroitin sulphate moiety on S201. The figure is based on (Gregers et al., 2003b; Strubin et al., 1986a).

can fit, and is open at the ends, allowing peptides of varying length to bind. In humans, there are three pairs of polymorphic MHC II genes, named human leukocyte antigen (HLA)-DR, -DP and -DQ (Trowsdale et al., 1991), and two pairs of non-classical MHC II genes, HLA-DM (Kelly et al., 1991) and HLA-DO (Karlsson et al., 1991). The genes are clustered on the short arm (q) of chromosome 6, one of the most gene-rich regions in mammalian genomes (1999). In the murine system, two major groups of the MHC II genes exist, I-A and I-E, as well as the non-classical genes encoding H-2M and H-2O. The Ii gene is located on human chromosome 5 (q32), and was first identified in 1979 by Jones *et al.* (Jones et al., 1979), when the MHC II α chain and Ii could be separated by 2D-gels. However, it was not until 1989 that Ii was found to have a role in antigen presentation (Stockinger et al., 1989). In 1995 the Leukocyte Typing Workshop decided that Ii should be designated CD74 (Moller et al., 1995). Ii is a type II TM glycoprotein which exists in different isoforms defined by the primary amino acid sequence. There are four isoforms of Ii in humans, p33, p35, p41 and p43. Ii p33 and p41 are distinguished by alternative splicing of the Ii transcript where the p41 isoform contains an extra exon (exon 6b) (O'Sullivan et al., 1987; Strubin et al., 1986a). These two isoforms yield two additional protein products due to an N-terminal cytoplasmic extension of 16 residues, which results from an alternative translation initiation site (O'Sullivan et al., 1987; Strubin et al., 1986b). Only the p33 and p41 isoforms are found in mice (the former as p31). The major human p33 isoform has an N-terminal cytosolic tail of 30 amino acids, a TM domain consisting of amino acid 31-56 and a C-terminal 160 residue luminal domain (Claesson et al., 1983; Koch et al., 1987; Lipp et al., 1987) (Figure 3).

Expression and regulation of MHC II and Ii

In the APC lineages MHC II gene expression is modulated as a function of their developmental stage (Reith and Mach, 2001). Maturation of conventional DCs is accompanied by a transiently elevated transcription followed by more or less complete silencing to maintain the peptide-MHC II complexes formed at the time and place of activation (Landmann et al., 2001), whereas pDCs maintain MHC II production and turnover in order to continuously present endogenous viral antigens in an activated state (Young et al., 2008). MΘs upregulate MHC II expression after IFN-γ activation and B cells abolish MHC II expression completely upon differentiation to antibody producing plasma cells. MHC II expression is also tightly regulated in non-hematopoietic cells, TECs rapidly loose MHC II expression if they are removed from their thymic environment, and in IFN-γ inducible cells, MHC II expression is subject to suppression by several cytokines (Reith et al., 2005). These tightly regulated expression patterns reflect a stringent control system with various cis- and trans-acting elements described (Benoist and Mathis, 1990; Glimcher and Kara, 1992). Subtle variations within the cis-acting elements in the genes give variable amounts of the individual gene products, with Ii being expressed at a significantly higher level than the MHC II isoforms (Landsverk et al. paper II). The expression of MHC II genes is dependent on a dedicated transcriptional co-activator called the MHC II trans-activator (CIITA) (Reith et al., 2005; Wright and Ting, 2006). CIITA controls the constitutive, maturation dependent expression of MHC II genes in APCs as well as the inducible expression in other cell types (Khalil et al., 2002; Nagarajan et al., 2002; Taxman et al., 2000; Westerheide et al., 1997). This regulator is uniquely devoted to regulating genes involved in antigen

presentation, and apart from MHC I, MHC II, Ii and HLA-DM/-DO, only nine additional targets have been identified, most of which have, or are likely to have, a role in antigen presentation (Krawczyk et al., 2008). Three different isoforms of CIITA (pI, pIII and pIV) resulting from the use of four different promoters have been described (Muhlethaler-Mottet et al., 1997). These isoforms are surprisingly restricted in their expression, with pI being the principal CIITA in Mθs, most DC subsets, including mouse $CD8\alpha^+$ and $CD8\alpha^-$ DCs, LCs, BMDCs, human CD11c+ DCs and LCs. pIII is the dominant isoform in B cells, pDCs in human and mouse and is also expressed by human monocyte derived DCs, whereas pIV is expressed in IFN-γ stimulated non-hematopoietic cells and Mθs (reviewed in (Reith et al., 2005)).

FOLDING AND ASSEMBLY OF MHC II AND Ii

After translation and translocation into the ER, Ii and MHC II associate into a nonameric $(\alpha\beta Ii)_3$ complex. The precise order of assembly is not clear, but it is likely that Ii initially self-associates into trimers prior to association with MHC II, either as preassembled αβ complexes or with single α and ß chains (Anderson and Cresswell, 1994; Dixon et al., 2006; Lamb and Cresswell, 1992; Roche et al., 1991). Ii trimer formation is driven by luminal (Bijlmakers et al., 1994; Gedde-Dahl et al., 1997) and TM trimerization motifs (Ashman and Miller, 1999). The luminal trimerization domain, consisting of residues 163-183, has an intrinsic capacity to trimerize (Wakeham et al., 2003) and assumes a cylindrical shape (Jasanoff et al., 1998). The TM trimerization domain consists of a hydrophilic patch of polar amino acids (Ashman and Miller, 1999) forming an α-helical structure which assembles into a left-handed coiled-coil trimer (Kukol et al., 2002). The sequence of the TM domain is highly conserved across species (Bremnes et al., 2000) and mutations in this region can lead to failure in formation of mature MHC II complexes and consequent inefficient antigen presentation (Ashman and Miller, 1999; Frauwirth and Shastri, 2001). Thus, both the TM and luminal domains of Ii are able to make trimers, but both seem to be required for proper Ii structure and function. All four human Ii isoforms can be incorporated into mixed trimers, but as p33/41 is expressed 4-5 fold higher than p35/p43, and p41/43 significantly less than p33/35, p33 containing trimers will dominate (Arunachalam et al., 1994; Lamb and Cresswell, 1992; O'Sullivan et al., 1987). The luminal domain of Ii forms an extended structure which is important for the interaction between Ii and MHC II. Ii interacts with the MHC II αß chains predominantly through a sequence comprising residues 86-104 (Freisewinkel et al., 1993; Romagnoli and Germain, 1994). This region (the MHC class II associated invariant chain peptide; CLIP) occupies the peptide-binding groove of MHC II and it is situated between the TM and luminal trimerization domains of Ii (Ghosh et al., 1995; Stumptner and Benaroch, 1997). The two independent trimerization domains in Ii may thus serve to impose some structure order onto the CLIP region that can facilitate the assembly of MHC II with Ii in the ER (Ashman and Miller, 1999). Ii thereby promotes the correct assembly of the MHC II heterodimers and prevents binding of peptides in the ER (Anderson and Miller, 1992; Lee and McConnell, 1995; Malcherek et al., 1995; Romagnoli and Germain, 1994; Schaiff et al., 1991; Sette et al., 1995). The CLIP peptide can be subdivided into two functional regions where the C-terminal segment (aa 92-105) occupies the peptide binding groove and a proline rich N-terminal segment (residues 81-91) binds outside the groove and has been suggested to

be important for the fast off-rate for CLIP after degradation of Ii (Kropshofer et al., 1995a; Kropshofer et al., 1995b). The removal of CLIP must necessarily precede binding of antigenic peptides, so CLIP should not have an overly high affinity for the peptide binding groove. However, the greatest variation in the polymorphic MHC II family is found in this domain, so the affinity of Ii for individual αß dimers naturally varies dramatically (Malcherek et al., 1995; Sette et al., 1995). In addition to the CLIP region, several additional MHC II interacting domains of Ii have been described. A proline (P) rich region (aa 82-87) situated just outside CLIP mediates binding to the HLA-DR β chain (Neumann and Koch, 2006; Siebenkotten et al., 1998; Stumptner and Benaroch, 1997), and also a region C-terminal to CLIP (aa 103-118) (Thayer et al., 1999) and the TM domain (Castellino et al., 2001) have been identified as MHC II interaction sites.

MHC II AND Ii IN THE ENDOSOMAL SYSTEM

After assembly in the ER, nonameric $(\alpha\beta Ii)_3$ complexes are transported to endosomal compartments for loading of peptides derived from internalized antigens (Figure 4). The exact route and traffic machinery involved in this transport has not been definitively established. Several reports suggest that the complex is transported directly from the trans-Golgi network (TGN) to the endocytic pathway (Bénaroch et al., 1995; Liu et al., 1998; Warmerdam et al., 1996), whereas other studies describe an indirect pathway via the plasma membrane (Bremnes et al., 1994; Henne et al., 1995; Ong et al., 1999; Roche et al., 1993; Wang et al., 1997). What is clear is that Ii plays an important role in this pathway. Mice lacking Ii exhibit impaired antigen presentation of intact protein antigen, but when fed antigenic peptides, they responded comparable or even superior to wt mice (Bikoff et al., 1993; Elliott et al., 1994; Viville et al., 1993; Zimmermann et al., 1999). Thus, Ii functions to bring nascent MHC II and internalized antigen together in an endocytic compartment containing proteolytic enzymes capable of processing both antigen and Ii itself.

THE ENDOSOMAL SYSTEM

The endosomal system consists of a dynamic network of membrane-enclosed organelles that differ in biochemical composition, cellular localization and morphology. The secretory pathway describes the outward flow of transmembrane and lumenal proteins from the ER, through the Golgi network to diverse intracellular organelles or the cell surface, whilst the endocytic pathway involves the inward flow of transmembrane proteins and cargo to lysosomes for degradation, or to early endosomes for recycling back to the cell surface (Behnia and Munro, 2005; Perret et al., 2005). Within this complex network proteins are continuously sorted in diverse directions, and the very nature of the compartments changes as organelles are formed, mature and consumed/dispersed.

Endocytosed cargo first enters early endosomes, which subsequently mature to late endosomes that finally fuse with lysosomes, generally considered the endpoint of the endocytic pathway (Luzio et al., 2007). In most cells this pathway is devoted to acquiring nutrients and building blocks (lipids, amino-acids and carbohydrates) for cellular metabolism and production of cellular components. The progression though this pathway is rapid, taking 5-15 minutes (Stoorvogel et al., 1991; Thilo et al., 1995), and entails the rapid acquisition of low pH and increasing amounts of active endocytic proteases (Honey and Rudensky, 2003). The identity

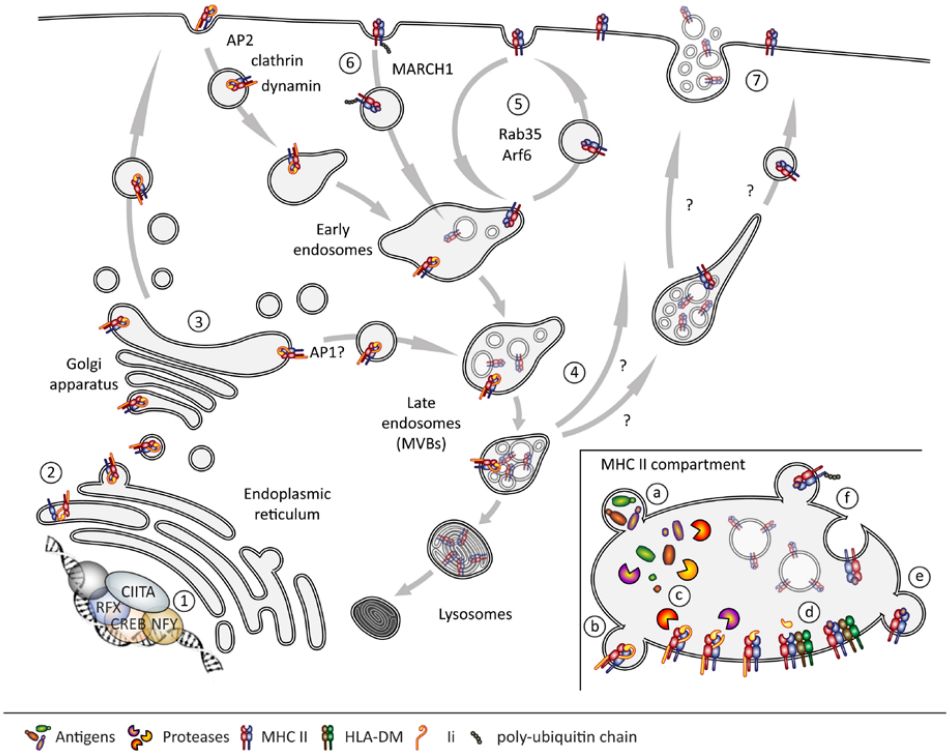

FIGURE 4 | MHC II AND Ii IN THE ENDOSOMAL SYSTEM. The endosomal system consists of morphologically, compositionally and functionally distinct compartments that are classified as early and late because they are sequentially accessed by endocytic cargo. As endosomes mature, they accumulate internal vesicles within them that are enriched in integral membrane proteins destined for lysosomal degradation. Late endosomes are also called multivesicular bodies (MVBs) and they receive newly synthesized lysosomal enzymes from the trans-Golgi network. Most MVBs fuse with lysosomes to degrade their contents, but in some cell types MVBs can also fuse with the plasma membrane and release their intralumenal vesicles as exosomes. MHC II and Ii genes are transcribed due to the action of the MHC II transactivator (1) and the translation products are inserted into the ER membrane where they assemble into nonameric complexes (2). From the trans-Golgi network Ii-MHC II can enter the endocytic pathway via the cell surface in an AP2 dependent manner, or possibly traffic directly to late endosomes (3). In endosomes Ii is degraded so that MHC II is freed to load peptides and traffic to the cell surface (4). Surface MHC II can recycle into Arf6 positive tubular recycling compartments (5) until they are ubiquitinated by specific ubiquitin E3 ligases (6) and sorted into intralumenal vesicles for degradation or release on exosomes (7). Endosomes accessed by MHC II are collectively termed MHC II compartments (MIICs). MIICs are accessible to endocytosed antigen (a) and new Ii-MHC II (b), and contain proteases which degrade both Ii and antigen (c). HLA-DM facilitates the exchange of CLIP for antigenic peptide (d) and peptide loaded MHC II can traffic to the cell surface (e). Mature MHC II is ubiquitinated by an ubiquitin E3 ligase and sorted into intra-lumenal vesicles for degradation (f).

of the different stages is maintained by specific lipid compositions and small GTP binding proteins (Behnia and Munro, 2005). Early endosomes have phosphatidylinositol 3-phosphate (PtdIns(3)P) in their membranes and are positive for Rab5, whereas recycling endosomes are positive for Rab4 and Rab11, and late endosomes contain domains enriched in Rab7a and Rab9 and the phosphoinositide PtdIns(3,5)P_2 (De Matteis and Godi, 2004; Stenmark, 2009). Lysosomes share several features with late endosomes, and the distinction between them is often unclear. Both compartments contain lysosome associated membrane proteins (LAMPs), lysosome integral membrane proteins (LIMPs) and endocytic proteases. But lysosomes lack the mannose 6-

phosphate receptors (M6PRs) required for protease transport, contain more active proteases, are more acidic and are generally more electron dense due to their multi-lamellar morphology (Saftig and Klumperman, 2009). Lysosomes are, however, heterogeneous organelles with hybrid organelles appearing post fusion with late endosomes and specific cell types contain lysosome related organelles, such as the lytic granules of cytotoxic T cells and NK cells, the melanosomes of melanocytes and the secretory granules of mast-cells (Blott and Griffiths, 2002; Luzio et al., 2007).

Membrane proteins destined for degradation in lysosomes are recognized by the endosomal sorting complex required for transport (ESCRT) machinery which mediates the budding of vesicles into the lumen of endosomes (Hurley and Hanson, 2010). Components of ESCRT-I and –II recognize mono- and/or Lys-63-poly-ubiquitinated cargo and cluster them into bud-like structures on the membrane, whilst ESCRT-III catalyses the scission of membrane necks and release of vesicles into the lumen (Duncan et al., 2006; Hurley, 2008; Wollert et al., 2009). The generation of intralumenal vesicles starts with the recruitment of Hrs to the endosomal membrane. Hrs has, in addition to its ubiquitin interacting motif, a FYVE domain (named after its 4 founding members: Fab1, YOTB, Vac1 and EEA1) which mediates binding to PtdIns(3)P on early endosomal membranes (Gaullier et al., 1998; Raiborg et al., 2002). The number of internal vesicles increases as endosomes acquire late endosomal characteristics, and after fusion with lysosomes, the internal membranes become densely packed and are ultimately degraded by lysosomal phospholipases (Schulze et al., 2009).

As endosomes mature, membranes and cargo is continuously retrieved backwards in the pathway and new components introduced. This entails the sequential assembly and disassembly of factors mediating budding and fission of vesicles from donor membranes, and tethering, docking and fusion with acceptor membranes. Budding of vesicles is initiated by adaptor proteins (APs) binding sorting motifs in the cytoplasmic tails of transmembrane proteins. APs recruit factors which, through hydrophobic insertions and scaffolding, generate curvature on membranes to facilitate the scission of transport vesicles by the concerted action of constricting factors and motor proteins pulling membranes along the cytoskeleton (Conibear, 2010). Vesicles can reach their destinations by the directed transport along the cytoskeleton. Kinesins and cytoplasmic dynein, moving toward the plus and minus end of microtubules, respectively, and myosins moving on actin filaments have all been implicated in such transport (Akhmanova and Hammer, 2010; Hammer and Wu, 2002). Reaching their target membranes, vesicles initially loosely adhere (tether) to allow for the pairing of soluble N-ethylmalemide-sensitive factor (NSF) attachment protein receptors (SNAREs) on opposing membranes (docking), which subsequently leads to membrane fusion (Cai et al., 2007; Jahn and Scheller, 2006). The fusion and fission of endosomes is regulated by Rab GTPases along with their cognate SNAREs. On early endosomes activated Rab5 recruits the phosphatidylinositol kinase Vps34 which generates PtdIns(3)P. The early endosomal antigen-1 (EEA1) binds to PtdIns(3)P and Rab5 on one membrane via its C-terminal FYVE and Rab5 interacting domains, and to incoming vesicles via a second N-terminal Rab5 interacting domain, thereby tethering two Rab5-positive membranes (Callaghan et al., 1999a; Callaghan et al., 1999b; Gaullier et al., 1998). EEA1 also interacts directly with early endosomal SNAREs

syntaxin-6 and -13 and facilitates their assembly and fusion (McBride et al., 1999; Simonsen et al., 1999a). Similarly the fusion between late endosomes and lysosomes or phagosomes seems to depend on Rab7a which recruits components of the homotypic vacuole fusion and protein sorting (HOPS) complex to tether late endosomal/lysosomal membranes together, and the SNARES syntaxin 7, syntaxin 8 and vesicle associated membrane protein (VAMP)-7 to drive the fusion (reviewed in (Luzio et al., 2007)).

Rabs also function in vesicle budding and motility through the recruitment of diverse effector proteins. Late endosomal Rab9 interacts with the sorting adaptor TIP47 and stimulates the budding of M6PR vesicles for retrieval to the trans-Golgi network (TGN) (Carroll et al., 2001), the recycling Rab11 interacts via the Rab11 family-interacting protein 2 (Rab11FIP2) with the actin based motor myosin Vb for transport of vesicles from recycling endosomes to the cell surface (Hales et al., 2002; Lapierre et al., 2001), and Rab5 has been shown to stimulate the association of endosomes with microtubules and their minus end directed movement (Nielsen et al., 1999).

TRAFFICKING OF Ii AND MHC II IN THE ENDOSOMAL SYSTEM

In humans, Ii trimers containing p35/43 are retained in the ER due to an arginine based ER retention signal contained in the 16 amino acid extension to the N-terminal cytosolic tail of these isoforms (Lotteau et al., 1990; Schutze et al., 1994). This retention can be bypassed through an interaction with the cytosolic tail of the HLA-DRβ chain (Khalil et al., 2003) and/or protein kinase C mediated phosphorylation of serine 8 in the Ii p35/43 cytosolic tail (Anderson et al., 1999; Kuwana et al., 1998), leading to 14-3-3β binding and blocking β-COP binding to the same site, thereby allowing for forward transport of p35/43 containing complexes (O'Kelly et al., 2002). Whether similar mechanisms exist in mice, which lack these isoforms, has not been determined. The cytoplasmic tail of all Ii isoforms from all species examined contain two di-leucine based sorting motifs which are essential for sorting MHC II heterodimers from the ER to endosomes (Bakke and Dobberstein, 1990; Bremnes et al., 1994). Both leucine signals can interact independently with AP-1 and AP-2, but not AP-3 *in vitro* (Hofmann et al., 1999; Rodionov and Bakke, 1998). AP-2 mediates transport between the plasma membrane and early endosomes, whereas AP-1 and AP-3 are involved in direct sorting from the TGN to late endosomes/lysosomes (Bonifacino and Traub, 2003; Sandoval and Bakke, 1994). In mice lacking the δ subunit of AP-3 MHC II trafficking in Mθs, lymphoblasts and splenocytes is normal (Sevilla et al., 2001), and the same was found for human B-lymphoblast cell-lines lacking AP-3 (Caplan et al., 2000). RNA interference targeting the various APs in model cells expressing MHC II, revealed that only depletion of AP-2 results in defective MHC II transport, with accumulation of MHC II-Ii complexes at the cell surface (Dugast et al., 2005; McCormick et al., 2005). This would indicate that the newly synthesized MHC II-Ii complexes are targeted to endosomes mainly via the plasma membrane, but whether this is the major pathway in APCs is still unclear. Silencing AP-1 in immature murine BM-DC does not compromise delivery of new MHC II-Ii to endosomes, but inhibition of AP-1 in mature BM-DCs results in accumulation of Ii at the cell surface (Santambrogio et al., 2005). This suggests that DCs differentially regulate the transport of new MHC II in response to pathogenic stimuli, with a shift toward more direct sorting to endosomes upon maturation.

Alternative entry levels of MHC II-Ii complexes into the endocytic pathway could promote the presentation of a broader spectrum of antigenic peptides by early loading of peptides susceptible to terminal degradation in late compartments and later loading of protease resistant epitopes. Such dual routing has been described for LAMP 1 (Carlsson and Fukuda, 1992), the lysosomal membrane glycoprotein lgp120 (Harter and Mellman, 1992) and M6PR (Johnson and Kornfeld, 1992). Transport of new MHC II to compartments containing BcR internalized antigen has been shown to depend on Ii and the actin based motor myosin II. Whether this is due to a direct interaction or via some unknown adaptor, and whether these MHC II-Ii containing vesicles originated from the cell surface or the TGN was not determined in this study (Vascotto et al., 2007).

PROTEOLYSIS AND MHC II PEPTIDE LOADING

In endosomes proteolysis of Ii occurs through sequential cleavage from the lumenal (C-terminal) side, generating cleavage products of approximately 22kDa, 10kDa, finally leaving only CLIP bound in the peptide binding groove of MHC II. The proteases involved in this processing have been the subject of intense studies for many years. It is now apparent that the endosomal proteases involved show somewhat overlapping specificities, a fact reflected by the divergent repertoires of these enzymes expressed by APCs (reviewed in (Hsing and Rudensky, 2005; Watts, 2004)). However, cathepsin S seems to play a central role as APCs from mice lacking this protease exhibit impaired MHC II transport with accumulated MHC II in endosomes and decreased motility due to defective Ii proteolysis (Driessen et al., 1999; Faure-Andre et al., 2008; Nakagawa et al., 1999). Intriguingly, Ii is itself able to modulate the activity of endocytic proteases. The Ii p41/43 isoforms contain a sequence in the alternatively spliced exon 6b which inhibits the activity of several cysteine proteases (Bevec et al., 1996; Fineschi et al., 1996; Lennon-Dumenil et al., 2001; Mihelic et al., 2008; Ogrinc et al., 1993), suggesting that this fragment can reduce proteolytic activity in endosomes and thereby protect antigenic epitopes from excessive degradation. Conversely, activated DCs and Mθs secrete cathepsin L complexed with a fragment of Ii p41/43 (exon 6b), and this enzyme is catalytically active in the extracellular milieu. Regulated secretion of the exon 6b stabilized cathepsin L was therefore proposed to facilitate migration of APCs by degrading the extracellular matrix (Fiebiger et al., 2002). Mouse strains expressing either Ii p31 or p41 were, however, found equally competent with respect to MHC II function and $CD4^+$ T cell development. These studies showed that Ii p41 could compensate for loss of p31 and converse, but whether the peptide repertoires expressed were similar was not determined (Takaesu et al., 1995; Takaesu et al., 1997). A somewhat surprising finding was that p41 but not p33 could to some extent compensate for lack of HLA-DM (se below) with regard to MHC II peptide loading, suggesting that CLIP derived from p41/43 might be less tightly bound to MHC II (Bikoff et al., 1998).

Exchange of CLIP for antigenic peptides is facilitated through the action of HLA-DM, which binds to MHC II and induces an altered conformation in the peptide binding groove, resulting in the release of CLIP and binding of high-affinity peptides (Busch et al., 2005). In B cells HLA-DM activity can be regulated by HLA-DO, which seems to promotes HLA-DM activity at low pH and restrict its activity at neutral pH. This has been proposed to promote the generation of high affinity antibodies by restricting

peptide loading on MHC II to antigens carried through to low pH compartments by high affinity BcRs (Brocke et al., 2002). Expression of HLA-DO is generally restricted to B cells and TECs, but certain DC subsets such as the LCs may express HLA-DO in an immature state, but down-regulate it in response to maturation stimuli (Hornell et al., 2006). HLA-DO expression correlates with increased levels of MHC II loaded with CLIP peptide at the cell surface in model cell lines and transduced moDCs (Bellemare-Pelletier et al., 2005; Denzin et al., 1997; van Ham et al., 1997).

Retrograde transport of mature MHC II

After proteolysis of Ii and peptide loading, *mature* MHC II (i.e. Ii disassociated, peptide loaded MHC II) traffic to the cell surface where their peptides can be sampled by $CD4^+$ T lymphocytes. The sorting signals and machinery involved in this step has not been resolved, however, peptide loading promotes a conformational change in MHC II which is sufficient for transport of MHC II to the surface (Germain and Hendrix, 1991; Neefjes and Ploegh, 1992; Sadegh-Nasseri and Germain, 1991; Thery et al., 1998; Wettstein et al., 1991). In a human melanoma cell line endosomes enriched in MHC II can move bi-directionally along microtubules, due to the opposing action of dynamin and kinesin, and these compartments were able to fuse directly with the cell surface (Wubbolts et al., 1999; Wubbolts et al., 1996). Fusion of endosomes with the cell surface has also been observed in human B lymphoblastoid cells and moDCs with the resulting release of lumenal vesicles, called exosomes, which contain both MHC II and co-stimulatory molecules, and can activate $CD4^+$ T cells. However, this occurs rarely, and is probably not a major pathway for surface delivery of MHC II (Kleijmeer et al., 2001; Raposo et al., 1996). In activated mouse BMDCs, endosomes containing MHC II can tubulate along microtubuli towards T cells in an antigen specific manner (Boes et al., 2003; Boes et al., 2002), and tubules or vesicles formed at their leading edge has been shown to fuse with the cell surface (Chow et al., 2002). Tubular extensions from multivesicular endosomes has also been observed upon activation of mouse spleen derived DCs and human moDCs (Barois et al., 2002; Kleijmeer et al., 2001), but as these tubules are absent from immature cells, the steady state transport of MHC II to the cell surface in immature DCs and other APCs probably occurs by vesicular transport involving some as yet unidentified components. In mice BMDCs and human B cells a compartment termed the class II vesicle (CIIV) has been proposed as a transport vesicle for peptide loaded MHC II. CIIVs contain MHC II loaded with peptides generated in late endosomal/lysosomal compartments, but lacks LAMP1, HLA-DM and Ii (Amigorena et al., 1994; Turley et al., 2000), however the relative large size, intralumenal vesicles and accessibility to immune complexes and the transferrin receptor is very suggestive of a recycling, early endosome.

MHC II at the cell surface

At the cell surface mature MHC II can recycle into recycling compartments and load, or exchange, peptides in a HLA-DM dependent or independent manner (Pathak et al., 2001; Pinet and Long, 1998). Recycling events may thereby contribute to the diversity of T cell epitopes presented. Recycling was originally proposed to be due to a leucine based sorting signal in the β-chain, thereby involving AP-2 and clathrin mediated endocytosis (Simonsen et al., 1999b; Zhong et al., 1997). However, the internalization of pMHC II was recently found to be independent of the cytosolic tails of MHC II, and depletion of AP-2, clathrin and dynamin had no

effect on this recycling (Walseng et al., 2008). In this study pMHC II was found to enter tubular early endosomes containing the ADP ribosylation factor (ARF)-6 and Rab35, two small GTPases which are involved in protein recycling (Donaldson, 2003; Kouranti et al., 2006). Whether these GTPases were involved in the internalization or return to the cell surface, and whether these compartments were the same or distinct from conventional recycling endosomes was not addressed in this study.

Mature MHC II (but not Ii associated) is also subject to ubiquitination by specific ubiquitin ligases acting on a conserved lysine residue in the cytosolic tail of the β-chain. (Ohmura-Hoshino et al., 2006b; Shin et al., 2006; van Niel et al., 2006). The E3 ubiquitin ligase membrane-associated RING-CH 1 (MARCH1) attaches a poly-ubiquitin chain to MHC II which presumably leads to ESCRT mediated sorting into intralumenal vesicles and degradation in lysosomes. In conventional DCs in mice and in human moDCs this ubiquitination is developmentally regulated. In an immature state DCs continuously exchange their MHC II, but upon maturation ubiquitination of MHC II ceases due to a the rapid silencing of MARCH-1 to prevent degradation of antigenic pMHC II complexes generated at the time and place of activation (De Gassart et al., 2008; Walseng et al., 2010a; Walseng et al., 2010b; Young et al., 2008). Another substrate of MARCH-1 is the costimulatory molecule CD86, so silencing this ligase also increases the expression of this co-stimulatory molecule required for T cell activation (Ohmura-Hoshino et al., 2009). Interestingly, MARCH-1 is upregulated in monocytes in response to IL-10, indicating that regulating MHC II turn-over is an important immune regulatory mechanism, but whether this is the case also in other APCs remains to be established (Thibodeau et al., 2008).

ANTIGEN UPTAKE AND TRANSPORT IN THE ENDOCYTIC PATHWAY.

Antigens can enter the cell via diverse endocytic mechanisms, creating endosomal compartments with distinct composition and functional properties (Doherty and McMahon, 2009). Receptor mediated endocytosis involves the specific recognition of a ligand and subsequent internalization via specific adaptors and coats depending on the internalizing receptor. Classical examples of such receptors are the BcR, Dectin-1, the scavenger receptor (SR) and the mannose receptor (MR). Phagocytosis is also initiated by receptor mediated recognition of invariant patterns and the activation of numerous cytoplasmic effectors, but involves actin driven membrane protrusion and particle engulfment (reviewed in (Underhill and Ozinsky, 2002)). The repertoire of receptors activated determines the functional maturation of the phagosome and the production and release of pro- or anti-inflammatory mediators (Blander and Medzhitov, 2006; Stuart and Ezekowitz, 2005). Macropinocytosis occurs without the need for prior recognition of cargo. Uptake of fluid phase antigens occurs as membrane protrusions fuse together, capturing any antigens trapped between them. This negates the necessity for specific receptor recognition, and allows for the presentation of peptides derived from previously unknown pathogenic entities (reviewed in (Norbury, 2006)).

The manner in which a particular antigen is internalized can significantly affect the subsequent intracellular routing, and the nature of the endocytic compartment accessed can give varying outcomes with regard to subsequent antigen presentation. As such, antigen targeted to the MR supplies a species of early endosomes devoted to cross-presentation on MHC I, whereas the SR delivers antigen to

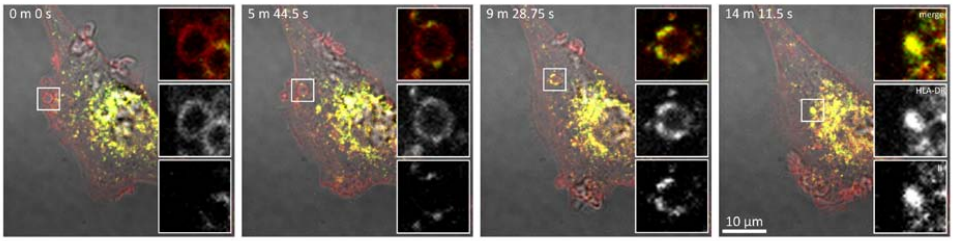

FIGURE 5 | MACROPINOCYTOSIS. Macropinocytosis occurs almost exclusively at sites of membrane ruffling. Actin driven protrusions enclose large volumes of the extracellular fluids and fuse together to form macropinosomes of up to 5 µm in diameter (Norbury, 2006). In human moDCs, these macropinosomes can be seen to interact extensively with endosomes containing both Ii and MHC II from their biogenesis at the PM, through to their consumption in perinuclear compartments. Selected images from a time-lapse sequence are presented, the insets shows one single tracked macropinosome in high magnification. Cells were pulsed with an alexa fluor 488 (Invitrogen, Carlsbad, CA, USA) labeled antibody targeting lumenal Ii (M-B741, BD biosciences, Franklin Lakes, NJ, USA), washed in RPMI and an antibody specific for mature MHC II (L243, BD biosciences, Franklin Lakes, NJ, USA) conjugated to alexa fluor 555 was added to the cell medium imediately before imaging. Confocal images were acquired on an Olympus FluoView 1000 inverted microscope equipped with a PlanApo 60/1.10 oil objective (Olympus, Hamburg, Germany). Constant temperature was set to 37°C and CO2 to 6% by an incubator enclosing the microscope stage. Images were processed in ImageJ (NIH, USA) and assembled in Adobe Illustrator (Adobe systems Inc., San Jose, CA, USA).

endosomes facilitating direct presentation on MHC II (Burgdorf et al., 2007). BcR internalized antigen transits via early endosomes to late endosomes, and end up in newly formed, BcR induced, multi-vesicular compartments (Lankar et al., 2002). As mentioned above, these events seem to be correlated with new MHC II-Ii trafficking. BcR ligation causes the activation of myosin II, which associates with Ii, causing the convergence of endosomes containing BcR internalized antigen and MHC II (Vascotto et al., 2007). Ii can also direct MHC II to phagosomes where it can be degraded, and MHC II freed to bind peptides (Ramachandra et al., 1999), but whether this occurs prior to fusion with lysosomes and generation of a phagolysosome is not clear. Macropinocytosed cargo is certainly readily made available to MHC II (West et al., 2004), and in DCs endosomes accessed by Ii and MHC II can be seen to interact extensively with these vesicles from their biogenesis at the PM, through to their consumption in acidic perinuclear compartments (Landsverk OJB, unpublished, Figure 5).

THE COMPARTMENT FOR PEPTIDE LOADING

Compartments enriched in MHC II are collectively termed MHC II compartments (MIICs). They were first described in 1991 by Peters *et al.* as compartments related to lysosomes, containing LAMP1 and CD63, but less acidic and distinct from late endosomes due to lack of M6PR (Peters et al., 1991). MIICs have subsequently been characterized morphologically as being multi-vesicular, multi-lamellar or electron-dense (reviewed in (Stern et al., 2006)). In an early electron microscopy study Kleijmeer and colleagues describe the accessibility of various MIICs to endocytic markers in B cells, MΘs, LCs and DCs from blood and spleen. They found that bovine serum albumin (BSA) was detectable in multivesicular MIICs within the first 10-30 minutes after uptake, and only appeared in multilamellar MIICs after 50 minutes. This led them to conclude that multivesicular MIICs were the precursors of multilamellar MIICs generated through maturation (Kleijmeer et al., 1997; Kleijmeer et al., 1996). This is highly reminiscent of the conventional endocytic pathway, however, lysosome related organelles

generated through endosomal maturation are found in other specialized cells and function in the storage and/or secretion of cell type specific components (Raposo et al., 2007).

MIIC MORPHOLOGY

The defining characteristic of the otherwise heterogeneous MIICs is the presence of large amounts of MHC II. It has been reported that MHC II alone can induce the formation of multilamellar MIICs and that this is dependent on the transmembrane and cytoplasmic domains of MHC II (Calafat et al., 1994). In view of recent studies describing the ubiquitination of MHC II and its role in the steady state turn-over of MHC II (Matsuki et al., 2007; van Niel et al., 2006; Walseng et al., 2010a), this indicates that ESCRT mediated sorting of MHC II onto internal membranes can generate the multi-vesicular/lamellar morphology of MIICs. As MHC II is necessarily relatively resistant to proteolysis due to its function to load peptides in proteolytic compartments, an intralumenal accumulation is to be expected, but how this promotes a multilamellar morphology is less clear. MHC II has, however, been shown to associate into both lipid and tetraspanin microdomains. In B lymphocytes the internal membranes of multivesicular MIICs are enriched in several tetraspanins (Escola et al., 1998; Hammond et al., 1998), and some of these are also present in MIICs from human moDCs and mouse BMDCs (Barois et al., 2002; Engering and Pieters, 2001; Vyas et al., 2007). The function of tetraspanins is thought to be linked to their ability to associate with one another and other proteins in an interacting network or 'tetraspanin web' (Charrin et al., 2009), and they might as such contribute to drive a multilamellar organization of internal membranes in the presence of MHC II. Interestingly one of these, CD63, is glucosylated during human moDC maturation, and this is accompanied by a change from multivesicular/multilamellar to densely packed membranes (Engering et al., 2003). However, the 'maintenance' of MIICs in B cells requires new protein synthesis, as incubation with cyclohexamide gradually decreases the number of MIICs (Calafat et al., 1994), and in moDCs maturation results in a decreased production of new MHC II and 'redistribution' of MHC II to the cell surface (Landmann et al., 2001)(Landsverk et al. paper II). The reduced amount of multilamellar MIICs could therefore be a consequence of less intracellular MHC II. Conversely, as monocytes differentiate to moDCs in response to IL-4 there is an increase in multilamellar MIICs (Potolicchio et al., 2005), but whether this is due to the increased expression of MHC II or other DC specific factors is unknown.

MIIC FUNCTION

The significance of accumulated intralumenal MHC II and multivesicular/lamellar morphology is poorly understood. MIICs were for a long time considered as a temporary storage compartment for delivering MHC II to the cell surface in response to pathogenic stimuli (Murk et al., 2002). This was based on the observation that in immature DCs MHC II was mainly present in endosomes whereas after activation it was strongly expressed at the cell surface (Cella et al., 1997). However, the decrease in MHC II ubiquitination (De Gassart et al., 2008; Shin et al., 2006; van Niel et al., 2006; Walseng et al., 2010b) and transient increase in new MHC II production (Villadangos et al., 2001) (Landsverk et al. paper II), strongly argues that this increase is at least in part due to new MHC II not being degraded (Villadangos et al., 2005).

The clear link between ubiquitination and MHC II degradation suggests that in the steady state these

compartments and their lumenal content are *en route* to lysosomal degradation. However, in human B cells multivesicular MIICs contain both CLIP associated- and peptide associated MHC II (Stang et al., 1998), and in human B cells and moDCs Ii is mainly present in MIICs with few internal vesicles, and barely detectable in multi-lamellar MIICs (Kleijmeer et al., 1996; Potolicchio et al., 2005). Thus, new MHC II clearly enters these same compartments in search for peptides. The essential peptide editing factor, HLA-DM, is present throughout the endocytic pathway, and the cell surface, but is particularly enriched in multivesicular, multilamellar and electron dense MIICs (Kleijmeer et al., 1997; Kleijmeer et al., 1996; Pierre et al., 1996), and HLA-DM assisted peptide loading has been shown in early compartments and even at the cell surface (Arndt et al., 2000; Santambrogio et al., 1999). However, open/empty MHC II and HLA-DM are predominantly detected in multilamellar compartments (Barois et al., 2002; Potolicchio et al., 2005), suggesting that peptide loading occurs primarily there. MHC II and HLA-DM are also strongly present on intralumenal membranes, and it has been suggested that intralumenal sorting of MHC II is an inherent part of the peptide loading process (Rocha and Neefjes, 2008). A fluorescence resonance energy transfer (FRET) based study found that HLA-DM and HLA-DR interact exclusively in the lumenal space of enlarged endosomes, and not in endosomes devoid of internal structures (Zwart et al., 2005). This entails the sorting of new MHC II into lumenal vesicles, HLA-DM assisted peptide loading and then back-fusion of lumenal vesicle with the limiting membrane. This poses an interesting problem, as the membrane topology and glycocalyx (carbohydrate parts of LAMPs and LIMPs, which serve to maintain the integrity of the limiting membrane), decreasing pH and increasingly proteolytic lumen of late compartments would require a novel machinery to drive back-fusion. Fusion of lumenal vesicles with the limiting membrane was proposed as early as 1988 (Griffiths et al., 1988), and has been proposed to contribute membrane for tubule formation in maturing DCs (Murk et al., 2002), but no factors capable of achieving this have been described so far, and in mice splenic DCs and human B cells HLA-DM is predominantly localized to the limiting membranes (Escola et al., 1998; Kleijmeer et al., 2001).

MIIC Formation

In most cells the endocytic pathway progresses rapidly from early endosomes, through late endosomes to lysosomes, whereas in B cells and DCs this pathway is delayed with accumulated late compartments (Kleijmeer et al., 1996). A likely scenario is that MIICs are diverted, albeit transiently, from the conventional rapidly maturing endocytic pathway. One potent candidate for contributing to MIIC diversion/biogenesis is the versatile Ii molecule (Stern et al., 2006). In paper I we show that in the presence of Ii mature MHC II accumulates in a slowly maturing endocytic compartment, and that this leads to a delayed degradation of membrane proteins sorted onto intralumenal membranes (Landsverk et al. paper I). Our lab has previously shown that Ii causes the delayed degradation of endocytosed cargo and proposed that this is due to a dramatic reorganization of the endocytic pathway induced by Ii (Gorvel et al., 1995a; Gregers et al., 2003c). In transfected human fibroblasts lacking MHC II, Ii induces the formation of enlarged endosomes which mature slowly from early endosomes through M6PR positive multivesicular endosomes and intermediate $M6PR^{pos}/LAMP1^{low}$ endosomes to dense $MPR^{neg}/LAMP1^{pos}$ lysosomes

(Stang and Bakke, 1997). Although these endosomes acquired intralumenal content, they did not to any great extent acquire the multilamellar morphology of classical MIICs, probably due to lack of MHC II, but were otherwise indistinguishable from the MIICs characterized by Kleijmeer and colleagues (Kleijmeer et al., 1996). These effects are dependent on the negative charges provided by acidic residues upstream of the leucine-based sorting signal in the cytosolic tail of Ii, and changing a single aspartic acid to arginine completely abolishes this effect (Nordeng et al., 2002). nuclear magnetic resonance studies followed by molecular dynamic simulations have shown that the α-helical Ii tails form a trimeric up-down-up or anti-parallel aggregate (Motta et al., 1997). It was therefore suggested that the cytosolic tails of Ii on opposing membranes could act as docking factors to promote endosome fusion, and this was demonstrated in a cell free endosome fusion assay (Nordeng et al., 2002).

The predominant presence of Ii in MIICs with few internal vesicles indicates that new MHC II-Ii enters endosomes at an early stage of MIIC formation (Kleijmeer et al., 1996; Potolicchio et al., 2005). As Ii is rapidly degraded in proteolytic compartments, and trimerization is necessary for the 'fusogenic properties' of Ii (Gedde-Dahl et al., 1997), the effect of Ii is probably restricted to the early events in MIIC formation. Indeed, in transfected cells, Ii induces the fusion of primarily early endosomes and fusion between late endosomes is rare (unpublished observations). Interestingly, these effects are not dependent on PtdIns(3)P and EEA1 (Nordeng et al., 2002)(Gregers *et al.* manuscript in preparation), factors normally required for early endosomal fusion (see above), suggesting that Ii alone can facilitate SNARE assembly. The generation of MIICs from early endosomes through endosomal fusion is not without precedence. In melanocytes, the biogenesis of melanosomes depends on factors which assemble SNAREs to drive early endosomal fusion, and these 'pre-melanosomes' subsequently mature and acquire late endosomal/lysosomal components such as LAMP1 and CD63 (Raposo et al., 2007). Intriguingly, the intracellular positioning (and proper functioning) of melanosomes depends on melanosome specific interactions with motor proteins for trafficking along microtubuli and the actin cytoskeleton (Raposo and Marks, 2007). Movement of MIICs along microtubules and actin filaments has also been observed, and the proper positioning of MIICs relative to BcR internalized antigen is critically dependent on an interaction between Ii and myosin II (Vascotto et al., 2007).

MIIC MATURATION

In paper I we show that Ii-induced enlarged early endosomes display a remarkably delayed maturation, with progression from early, Rab5 positive endosomes to late, Rab7a positive endosomes taking as long as 1-2 hours (Landsverk paper I). A similar enlargement of early endosomes can be induced by over expressing Rab5 or its GTPase deficient variant Rab5Q79L (Gorvel et al., 1991; Stenmark et al., 1994), but these endosomes mature rapidly, loosing Rab5 within 15-30 minutes, showing that increasing fusion alone does not delay maturation (Landsverk paper I). This delayed maturation is not dependent on the presence of MHC II, as a similar slow maturation is observed in cells not expressing MHC II (unpublished observations), suggesting that the presence of Ii alone diverts Ii-containing endosomes from the conventional endocytic pathway. How Ii achieves a delayed maturation is not clear. Conversion from early to late endosomes in the conventional

endocytic pathway has been proposed to involve the displacement of the Rab5 guanine nucleotide exchange factor (GEF) Rabex 5 by SAND-1/Mon1, which also drives the recruitment of Rab7a, possibly by interacting with the Rab7a GEF HOPS. How SAND-1/Mon1 is regulated is less clear, but has been proposed to involve sensing of endosomal size, the concentration of PtdIns(3)P or a combination of the two (Poteryaev et al., 2010). In transfected cells, Ii-induced endosomes can grow to a large size and endosomes with a diameter of up to 5-10µm are frequently seen at high levels of expression (unpublished observations), and a PtdIns sensor (2xFYVE) is strongly recruited to early Ii endosomes indicating that if the proposed mechanism of SAND-1/Mon1 is valid, Ii must be preventing its recruitment. Alternatively, Ii might be influencing other processes potentially involved in endosomal maturation, such as the recruitment of Rabs, GEFs or GTPase activating proteins (GAPs), assembly of SNAREs, recruitment of acidifying proton pumps or alkalizing NADPH oxidases, membrane lipid flipases, LAMPs or LIMPs. The components involved in regulating maturation in the conventional endocytic pathway and which factors Ii influences clearly requires further investigation. However, it seems likely that Ii is one factor contributing to transiently diverting the endocytic pathway from a rapid to a slowly maturing pathway. Through the maturation, these compartments continue to exchange content and membranes with incoming endosomes from the cell surface, thus continuously receiving both old MHC II and antigen (Landsverk et al., 2010b). MHC II-Ii is also recruited to phagosomes and macropinosomes (Ramachandra et al., 1999; West et al., 2004), so it is likely that these compartments are similarly influenced by Ii. Ii could thereby serve not only to bring MHC II and antigen together in an alternate endocytic pathway, but also contribute to the diversity of peptides generated and allow ample time for MHC II to sample them.

MIIC Diversity

It should be noted, however, that the properties of MIICs will vary depending on the particular cell type in question and also within the single cell depending on other factors recruited to the specific MIIC. For example Mθs have a particularly aggressive endocytic environment due to their functions in non-inflammatory removal of debris, apoptotic cells and the destruction and clearance of invading pathogens. As such, Mθs have low amounts of intracellular MHC II, possibly due to its rapid degradation in the steady state. Upon IFN-γ/LPS induced activation however, Mθs can decrease this proteolytic activity (Yates et al., 2007). IFN-γ stimulation of Mθs also leads to increased expression of Ii and MHC II, through induction of CIITA pIV expression (Pai et al., 2002), thus potentially contributing to enhanced MIIC biogenesis and decreased proteolysis of both antigen and MHC II. DCs, in contrast, generally have a relatively mild proteolytic endocytic environment, but moDCs in particular exhibit increased levels of lysosomal proteolysis as compared to other human dendritic cell populations (McCurley and Mellman, 2010), indicating that there may be some subset specific variation. These variations reflect the activation induced or steady state recruitment of additional components to MIICs, such as proteolytic enzymes (Lautwein et al., 2002; Lennon-Dumenil et al., 2002), protease inhibitors (El-Sukkari et al., 2003; Kitamura et al., 2005; Pierre and Mellman, 1998), vacuolar proton pumps (Trombetta et al., 2003) or NADPH oxidase (NOX)-2, which produces reactive oxygen species leading to decreased acidification (Savina et al., 2006; Savina et al., 2009). Thus, a MIIC may vary

significantly depending on the endocytic mechanism, the various additional components expressed/recruited and the APC type and activation stimuli. However, we propose that the underlying framework of MIIC biogenesis is, at least in part, undertaken by the endosome modulating functions of Ii.

Additional functions of Ii

Ii and B cell maturation

It should be mentioned that Ii has been shown to have additional functions apparently unrelated to MHC II transport and MIIC biogenesis. A 42-44 amino-acid long fragment of the N-terminal domain of Ii has been shown to be involved in B cell maturation and survival (Matza et al., 2003; Matza et al., 2002; Shachar and Flavell, 1996). This fragment is generated through cleavage of the trans-membrane segment of Ii by the γ-secretase/ presenelin complex (Becker-Herman et al., 2005), and has been shown to translocate to the nucleus and induce NF-κB activity through its effect on the trans-activation domain of p65/RelA (Becker-Herman et al., 2005; Matza et al., 2001).

Ii as a cell surface receptor

This was more recently linked to another function of Ii where it was shown to act as a cell surface receptor for macrophage migration inhibitory factor (MIF) (Gore et al., 2008; Leng et al., 2003), a cytokine involved in micro vascular and atherogenic recruitment of mononuclear cells (Noels et al., 2009). Signal transduction upon MIF binding to Ii requires CD44 (Meyer-Siegler et al., 2004; Shi et al., 2006), a cell-surface glycoprotein involved in cell-cell interactions and adhesion to the extracellular matrix (Johnson and Ruffell, 2009). MIF can also bind to chemokine receptors CXCR2 and CXCR4. CXCR2 has been co-immunoprecipitated with Ii, and both Ii and CXCR2 were found to be required for monocyte recruitment during inflammation (Bernhagen et al., 2007).

Ii and CD1 Transport

Ii has been implicated in the transport of MHC I-like CD1 family proteins which function to present lipid antigens to T cells (Salio et al., 2010). Ii has been co-immunoprecipitated with CD1a (and CD9 a tetraspanin involved in cell adhesion and migration) in immature moDCs, this association depends on lipid rafts and facilitates recycling of CD1a which lacks its own internalization motif (Sloma et al., 2008). However, CD1a has been shown to recycle in a Rab22a/ARF6 dependent pathway in Hela cells which do not express Ii, and also in these cells a fraction of CD1a partitions into lipid rafts (Barral et al., 2008). Ii has also been shown to associate with CD1d in B cells and this serves to direct CD1d into the endocytic system (Jayawardena-Wolf et al., 2001). In human moDCs Ii leads to enhanced presentation of exogenous (but not endogenous) antigens on CD1d (Chen et al., 2007). CD1d contains its own tyrosine based internalization motif for rapid recycling at the cell surface, and the enhanced antigen presentation in the presence of Ii presumably reflects a sorting into more proteolytic antigen processing compartments. Thus Ii might play a dual role in both sorting and MIIC biogenesis also for antigen presentation by CD1 proteins.

Ii and CD70 Co-Stimulation

The stimulus provided by co-stimulatory molecules is essential for the activation of MHC reactive T cells. The B7 family molecules CD80 and CD86 interact with CD28 or CTLA-4, and act as positive or negative regulators of T cell expansion, respectively (Greenwald et al., 2005). TNF receptor family

molecules OX40 and CD27 interact with OX40L and CD70, respectively, and induce a distinct signaling pathway which may break tolerance and induce differentiation of memory T cells (Denoeud and Moser, 2010; Redmond et al., 2009). Intriguingly, CD70 seems to rely on Ii for its transport to late endocytic compartments. Co-immunoprecipitation experiments show that CD70 associates with Ii in Meljuso cells and transfected Hela cells, and this might serve to coordinate delivery of MHC II with co-stimulatory ligands to distinct membrane domains upon APC activation (Zwart et al., 2010). This is an enticing concept, as APCs express a plentitude of self peptides on their MHC II in the steady state, restricting co-stimulation to MHC II from MIICs delivered to the cell surface after activation might reduce the potential for activating escaped self-reactive T cell clones. Whether similar mechanisms exist for the B7 family members is not known, but in immature DCs ubiquitin ligases acting on MHC II also target CD86 (Ohmura-Hoshino et al., 2006a), this would presumably likewise deliver CD86 to the intralumenal vesicles of MIICs, and could after reception of a maturation stimuli similarly deliver CD86 to membrane domains.

Ii, MHC I and FcRn Transport

Ii has also been found to associate with peptide free MHC I and somewhat surprisingly cause the increased cell surface expression of certain MHC I alleles (Powis, 2006; Reber et al., 2002). This is contrary to its action on all other partners and clearly requires further examination. Ii has also been co-immunoprecipitated with the MHC I related neonatal Fcγ Receptor (FcRn) in transfected Hela cells, and this association apparently leads to the relocation of FcRn to LAMP-1^{+} late endosomes/lysosomes (Ye et al., 2008).

Ii and Cancer

Ii is not surprisingly expressed in a variety of hematopoietic cancers, but is also present in cancers of the bladder, prostate and stomach, in some cases without the coordinate expression of MHC II (Meyer-Siegler et al., 2006; Meyer-Siegler et al., 2005; Tamori et al., 2005). The increased expression of Ii has been linked to poor clinical prognosis in patients with myeloid leukemia, multiple myeloma, non-Hodgkin's lymphoma, sarcoma, colon and gastric cancers (Chamuleau et al., 2004; Clements et al., 1992; Cuthbert et al., 2009; Ishigami et al., 2001; Jiang et al., 1999; Stein et al., 2004). These pro-carcinogenic effects of Ii have been linked to its role as a cell surface receptor (se above), where MIF binding to CD44-Ii leads to the activation of intracellular signaling pathways leading to cell proliferation and survival (reviewed in (Berkova et al., 2010)). The gram-negative bacterium *Helicobacter pylori* is strongly associated with gastric cancers. *H pylori* binds to Ii on gastric epithelial cells and induces MIF production, it might thereby contribute to enhanced survival of the hosting epithelial cells in infected mucosa (Beswick and Reyes, 2009). However, also the conventional properties of Ii could contribute to tumor development by promoting immune evasion of cancerous cells by preventing the presentation of endogenous tumor antigens (Humphreys et al., 2004).

Ii in Immunotherapy

In accordance with its wide distribution in a range of tumors and its function in antigen presentation, Ii has become an attractive target for cancer therapy. SiRNA mediated knock down of Ii has been used as an approach to enhance anti-tumour immunity (Ke et al., 2007; Qiu et al., 1999; Xu et al., 2004), and

antibody targeted cytotoxic drugs have proved highly effective in eliminating human tumor xenografts in monkeys and mice (Chang et al., 2005; Griffiths et al., 2003; Sapra et al., 2005). Milatuzumab, a humanized mouse-anti Ii antibody, is currently being evaluated in clinical trials for treating multiple myeloma, non-Hodgkin's lymphoma and chronic lymphocytic leukemia in humans (reviewed in (Berkova et al., 2010; Mark et al., 2009)). Ii is also being exploited as a targeting vector for delivering peptides to MHC II. Genetic exchange of CLIP with antigenic peptides has proven to be highly efficient in targeting peptides into the peptide binding groove of MHC II (Fujii et al., 1998; Malcherek et al., 1998; Nakano et al., 1997; Van Bergen et al., 1997), and even low affinity peptides that do not efficiently bind to MHC II have been shown to be capable of activating CD4+ T cells (Carstens et al., 2000; Gregers et al., 2003a). DNA vaccines based on peptide/CLIP replaced Ii genes have been shown to elicit specific T cell responses, induce protective immunity against infections (Nagata et al., 2002; Nagata et al., 2001; van Tienhoven et al., 2001), and anti-tumour responses in animal models (Brulet et al., 2007; Gao et al., 2006). Thus using Ii as a vehicle for loading peptides onto MHC II might be a promising approach to elicit pathogen/tumour specific responses in cases where the target sequence is known.

AIMS OF THE THESIS

Presentation of exogenous antigen to naïve T cells requires the coordination of diverse intracellular events to achieve the uptake, processing and loading of peptides on MHC II molecules. In APCs in general and DCs in particular the endocytic pathway is adapted to serve this purpose, and exhibits dynamic alterations in response to pathogenic challenge. This thesis aims to characterize the role of Ii in modulating the endocytic pathway in the steady state to promote MHC II antigen presentation, and investigate the molecular basis for an Ii mediated modulation during DC maturation.

The presence of Ii causes a delayed degradation and processing of endocytosed antigens, and leads to the fusion of MHC class II containing vesicles (Gorvel et al., 1995b; Gregers et al., 2003c; Nordeng et al., 2002). When Ii is over-expressed in experimental systems these eventually form large endosomal vesicles and these properties have been shown to be dependant on particular domains within the cytosolic tail of Ii (Nordeng et al., 2002). In the steady state, APCs contain lysosome related organelles (MIICs) that are accessed by both old and newly synthesized MHC II (Landsverk et al., 2009). The role of Ii in directing new MHC II to MIICs is firmly established, but how old MHC II is sorted to MIICs is unknown. To compare and discriminate between the primary role of Ii in new MHC II assembly and transport, and a secondary role on mature MHC II, we designed a novel experimental system combining photo activation and induced expression. Our findings were recently published in Immunology and Cell Biology (Landsverk et al., 2010b) and confirm a role for Ii in the biogenesis of a compartment receiving both old and new MHC II.

DCs undergo remarkable phenotypical and functional changes upon pathogen or cytokine induced activation. The endosomal system undergoes severe re-organization, with modulated proteolytic capacity and dynamic tubule formation. Migration is transiently halted to accommodate antigen uptake (Faure-Andre et al., 2008) and MHC II loading and transport to the cell surface is enhanced. As Ii can affect all these processes we sought to determine the transcriptional/translational rational for a role for Ii in these processes. By quantitative real-time PCR and biochemical approaches we examined induced responses to pathogenic stimuli and inflammatory cytokines. Our findings presented in paper II indicate that the presumption that all CIITA controlled genes are equally affected upon maturation is not valid, and a large pool of functional Ii is maintained in mature DCs.

SUMMARY OF INCLUDED PAPERS

PAPER I. INVARIANT CHAIN INCREASES THE HALF-LIFE OF MHC II BY DELAYING ENDOSOMAL MATURATION. In steady state APCs MHC II is most often found accumulated in late endosomes containing LAMP1 and CD63. Within these compartments, MHC II is particularly accumulated on the intralumenal vesicles, several studies have confirmed a role for ubiquitin ligases in the degradation of MHC II (Matsuki et al., 2007; Ohmura-Hoshino et al., 2006b; Shin et al., 2006; van Niel et al., 2006; Walseng et al., 2010a), suggesting that mature MHC II is sorted onto intralumenal vesicles via the conventional ubiquitin-ESCRT pathway for degradation of membrane proteins. Importantly, Ii associated MHC II is not ubiquitinated (van Niel et al., 2006). During peptide loading MHC II is dissociated from Ii through proteolysis and the action of DM and mature MHC II is freed to traffic to the cell surface and present its antigenic peptides to CD4+ T cells. Mature, cell surface MHC II can recycle into Arf6 and Rab35 positive tubular endosomes, and this internalization is independent of AP-2 and clathrin (Walseng et al., 2008). However, Ii-associated MHC II is dependent on AP-2 and clathrin for its trafficking to peptide loading compartments (Dugast et al., 2005; McCormick et al., 2005). In our studies of the role of Ii in MHC II trafficking we have consistently observed an accumulation of MHC II into endosomes induced by Ii. In paper I we used a conformation specific antibody and disclosed that also mature MHC II is directed to these endosomes. Moreover, these compartments acquired a multivesicular morphology with abundant MHC II present on intralumenal vesicles. To conclusively determine a role for Ii in the sorting of mature MHC II we designed a novel experimental system combining a photo-activable green fluorescent protein (PAGFP) attached to MHC II and an inducible expression system for Ii. This allowed us to image a sub-population of photo activated MHC II, and examine how they were distributed to endosomes in response to newly produced Ii. This redistribution was not dependent on a 'secondary' interaction of mature MHC II with new Ii, or the sorting/ubiquitination motifs in the cytosolic tails of MHC II, and did not affect the total amount of MHC II expressed at the cell surface. We did, however, find that in the presence of Ii, the half life of MHC II was increased and that this was due to a greatly delayed maturation of Ii containing endosomes. Thus we conclude that Ii induces an APC specific endocytic environment where endocytic cargo such as MHC II and antigen are converged and conserved in order to promote the proteolysis, loading and presentation of a diverse array of antigenic peptides to T cells (Landsverk et al., 2010b).

PAPER II. DIFFERENTIAL REGULATION OF MHC II AND INVARIANT CHAIN EXPRESSION DURING MATURATION OF MONOCYTE DERIVED DENTDRITIC CELLS. This study was initiated in order to examine the transcriptional and translational basis for diverse Ii functions during the maturation of DCs. Several studies have attributed functions to Ii during the activation of DCs. Fiebiger et al. found that a fragment of Ii p41 could stabilize extracellular cathepsin L, possibly to achieve the degradation of extracellular matrix and facilitate the influx of effector cells to inflammatory sites (Fiebiger et al., 2002), whereas a 1998 Cell paper attributed the regulation of cell surface MHC II to the efficiency of Ii processing by cathepsin S and ultimately its inhibitor cystatin C (Pierre and Mellman, 1998).

Most notably, Faure-André et al. in an elegant Science paper describe a pause in DC migration upon activation due to the transient binding up of myosin II by elevated Ii (Faure-Andre et al., 2008). In our pursuit of an understanding of the functions of Ii on the endomembrane system and its possible modulation during pathogen encounter, we wanted to examine in detail the regulation of Ii as compared to MHC II during DC maturation. Most previous studies have only examined the transcription of MHC II and presumed that Ii follows a similar course due to its mutual dependence on CIITA. Interestingly, we found that the regulation of Ii was not strictly correlated with that of MHC II. Ii mRNAs were significantly less upregulated at early time-points after activation, and decreased more slowly at later time-points. This indicates that the turn-over of CIITA at Ii promoter binding sites was lower than at the MHC II loci and less influenced by external stimuli. Alternatively, Ii mRNA was more stable than that of MHC II. The levels of mRNA are not necessarily a good measure of gene output due to their differential stability and affinity for ribosomal proteins, we therefore performed metabolic labeling and chase experiments on immature and mature DCs to examine new protein synthesis and half-life. Our findings corroborated and expanded the qRT-PCR data, showing that in mature cells there is an abundance of Ii, mostly non-associated with MHC II. Immunofluorescence microscopy indicated that the majority of this population resided in the ER, but biochemical assays showed that Ii gained access to endosomes and was degraded also in mature DCs. In view of our results presented in paper I and previous findings (Gregers et al., 2003c; Nordeng et al., 2002), this suggests that Ii could play a role in modulating the endocytic pathway in mature DCs. We find that there is a biosynthetic rational for diverse Ii functions also in mature DCs, and suggest that Ii could facilitate the modulation of endosomal compartments for preserving antigen for loading on recycling MHC II or transfer to lymph node resident DCs after entry into these organs.

METHODOLOGICAL CONSIDERATIONS

MICROSCOPY

Microscopes have been vital tools for scientists since their development at the end of the sixteenth century and modern imaging technology has greatly expanded the scope of questions we can approach. At its core, microscopy is all about deriving an image from the interaction of light with matter. In fluorescent microscopy, fluorophores with distinct excitation and emission spectra can be distinguished within the same sample by applying light with specific wavelengths and collecting the emitted light (Lichtman and Conchello, 2005). Fluorescently labeled antibodies were first introduced in 1941 (Coons et al., 1942) and now fluorophores with a color spectrum ranging from ultra-violet to infra-red are available for labeling molecules and organelles. For sub-cellular imaging in real-time, fluorophore-conjugated antibodies have some limitations due to the large size (e.g. 150kDa for IgG) and membrane impermeability of antigen targeting moieties. The advent of fluorescent proteins revolutionized science by allowing the specific labeling of distinct proteins *in vivo* and their visualization in both space and time. The green fluorescent protein (GFP) was first used as a fluorescent probe in 1994 (Chalfie et al., 1994), and in 2008 Osamu Shimomura, Martin Chalfie and Roger Y. Tsien were awarded the Nobel prize in chemistry for its discovery and development. Today related fluorescent proteins covering nearly the entire visual spectrum are available (Chudakov et al., 2010), and their use extends beyond resolving

molecular processes in space and time, to studies of protein function, dynamics and interactions (Wang et al., 2008).

In paper I (and (Bergeland et al., 2008)) we use a photo-activable GFP (PAGFP) which was designed to enhance the inherent photo-convertible properties of the original GFP (Patterson and Lippincott-Schwartz, 2002). PAGFP has a barely detectable absorbance of 488nm light in its native state, but upon exposure to ultra-violet light, PAGFP gains a 100-fold increase in fluorescence emission (517nm) upon 488nm excitation (Figure 6). The photo-conversion involve an irreversible shift in the chromophore from a neutral phenolic form to an anionic phenolate form (Patterson and Lippincott-Schwartz, 2002). This enables the selective labeling in time and space of a population of PAGFP tagged molecules (figure 6), and allowed us to examine the effect of induced Ii on exclusively old MHC II (Landsverk et al., 2010b). However, although PAGFP is only 2 aa (S65T, T203H) removed from enhanced GFP (EGFP), it has an almost three-fold reduced molar extinction coefficient (ε) and barely half the brightness. Importantly, PAGFP is significantly less photo-stable and is rapidly photo-bleached, consequently, long time imaging requires a low intensity laser and low resolution scanning. In paper I (and (Landsverk et al., 2010a)), we also use the photo stabilized, enhanced, GFP (EGFP) and the monomeric (m-) Cherry. Although controls were performed for all the fusion constructs, it should be noted that attachment of a large (220-240 aa, 25kDa) β-barrel to *any* protein could interfere with its normal function.

The use of microscopes and fluorescent probes has undoubtedly revolutionized life sciences, however, the acquisition, analysis and interpretation of microscopy data is challenging. Fluorophores have excitation and emission spectra which can absorb and emit light along its entire spectrum and this makes crosstalk (bleed-through) an issue. Equally problematic is the overlap of specific fluorescence

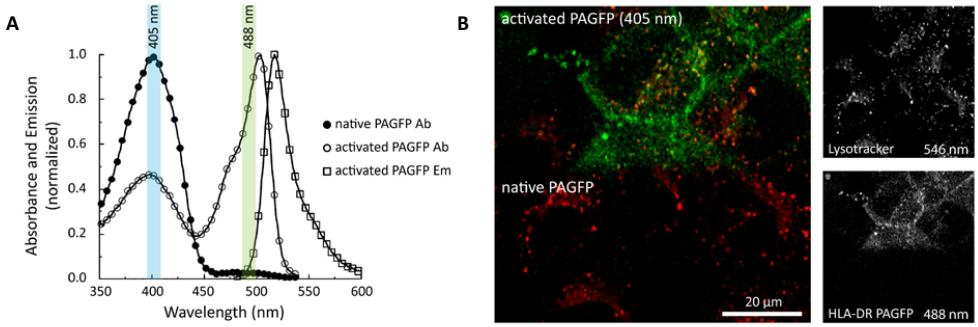

FIGURE 6 | PAGFP ABSORBANCE AND EMISSION SPECTRA. A) Native PAGFP optimally absorbs light of ca 400 nm wavelength (solid circles) and undergoes an irreversible photo-conversion to a GFP-like absorption spectrum (open circles) with a resulting 100-fold increase in fluorescence emission (517nm, open squares) upon 488nm excitation. Graph from (Patterson and Lippincott-Schwartz, 2004). B) Madin Darby canine kidney cells stably transfected with HLA-DR1 alpha fused to PAGFP and HLA-DR1 beta (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) were incubated with lysotracker red (Invitrogen), and PAGFP was activated in half the frame with a 405 nm laser pulse. Confocal images were acquired on an Olympus FluoView 1000 inverted microscope equipped with a PlanApo 60/1.10 oil objective (Olympus, Hamburg, Germany). Lysotracker red was excited with 546 nm and PAGFP with 488 nm, excitation DM405/488/559/635, spectral emission filters SDM560 (500-545) and SDM640 (570-630). Constant temperature was set to 37°C and CO2 to 6% by an incubator enclosing the microscope stage. Images were processed in ImageJ (NIH, USA) and assembled in Adobe Illustrator (Adobe systems Inc., San Jose, CA, USA). One single image from a time-lapse sequence is shown, in red lysotracker, in green activated PAGFP.

with background autofluorescence. Autofluorescence spectra are generally broad and encompass most of the visible spectral range, overlapping the excitation/emission wavelengths of GFP and its many derivatives, and this can lead to low signal-to-noise ratio. Unfortunately, autofluorescence is not uniformly distributed, and organelles such as mitochondria and lysosomes tend to have a higher autofluorescence than their surroundings. With increasingly sensitive detection systems (charge-coupled devices (CCDs), or photomultiplier tubes (PMTs)), these signals can easily be mistaken for specific. New fluorophores with narrow, non-overlapping excitation/emission spectra, fine-tuned lasers, excitation filters, spectral emission filters and sequential scanning can reduce such artifacts, but requires insight into the physical aspects of fluorophores, and microscope settings applied (North, 2006). In fluorescent microscopy, any acquired signal should be dissected in order to determine its correct origin.

In paper I we worked for a long time after the hypothesis that Ii might capture old MHC II at the cell surface and redirect it to MHC II compartments. This hypothesis, as many in our laboratory and elsewhere, was based on observations made at the microscope. We observed that with increasing levels of Ii, MHC II was mainly present in enlarged endosomes and apparently depleted from the cell surface. However, despite a large body of alternative experimental approaches, we could not detect any complexes formed between old MHC II and new Ii, nor a decrease in total surface MHC II. These findings eventually led to a revisal of the hypothesis and the story presented in paper I (Landsverk et al., 2010b). In our initial observations we were deceived by high intensity staining of endosomal MHC II in cells expressing Ii. To avoid over-exposure we adjusted our acquisition parameters and gained endosomal resolution, but concomitantly a weaker signal from the less concentrated cell surface pool of MHC II. Such is modern microscopy, biological events that alter the properties of our specimens can be compensated for (or disguised) by adjusting laser intensities, PMTs/CCDs and software to fit our predisposed conclusions. As visually predisposed beings we seek resolution and adjust our settings to resolve elements with high signal at the cost of elements with low intensity, but quite possibly of equal or greater relevance. Unbiased acquisition and analysis of microscopy data would be optimal, but the automated imaging systems need to overcome several obstacles before they be widely applied to produce reliable data. Analytical software struggles to distinguish the heterogeneous features and natural variation in biological specimens, and manual definition of acquisition and analysis parameters is still required.

The advances in fluorescent probes and microscope technology with better lasers, filters, PMTs/CCDs and software allows for manipulation of real and artifactual signals. Correct interpretation of visual data necessitates an in depth knowledge of the processes examined and the technical experience to evaluate the integrity of acquired data and its biological significance. Users and analysts should be aware of the pitfalls and exhibit critical judgment in the interpretation of any microscopy data, quantified or not. Seeing is believing, but your eyes (or software) may be deceiving you.

CELL LINES AND IN VITRO GENERATION OF DCS

In paper I we used human fibroblasts, melanoma cells and canine kidney cells to examine the impact of Ii on old MHC II. These cells lines are not APCs, and with the exception of the Meljuso cells, do not

express endogenous MHC II, Ii nor other CIITA target genes or lineage specific genes induced during APC development. However, MHC II expression can be induced in various cell types, including fibroblasts, in response to IFN-γ which leads to CIITA pIV expression, sufficient to achieve MHC II antigen presentation. CIITA regulates only a small number of genes, and apart from MHC II, HLA-DM, HLA-DO and Ii, only nine other genes are regulated. These include an ubiquitin E3 ligase (TRIM26), an alternative isoform of the early recycling Rab4 (Rab4b) and an immunoglobulin family member (MYBPC2) which can bind myosin and actin, as well as a lysosomal protease (TPP1) (Krawczyk et al., 2008). This suggests that the minimal requirement for MHC II antigen presentation involves few additional factors. The experimental system we have used with selective transfection of a few genes into non-APCs enable us to define the role of Ii in a setting where no other APC specific factors are present, and thereby avoids any 'contamination' of the results by unidentified/uncharacterized elements.

In paper II we examine the molecular basis for Ii function in maturing moDCs. The heterogeneity within the DC network and the development of DC-like cells from monocyte precursors during inflammation is outlined in the theoretical discussion. However, the in vitro differentiation of DCs from human monocytes is far removed from moDC development during inflammation in vivo. Human, 'immature', moDCs differentiated in the presence of GM-CSF and IL-4 presumably represent an intermediate stage between monocytes and the inflammatory DCs observed in mice, with a full DC phenotype acquired upon activation with PRR ligands or inflammatory cytokines. Monocytes can also give rise to other functionally distinct moDC types depending on the cytokines they are exposed to during differentiation. For example culture in GM-CSF and IL-15 generates moDCs which are superior at inducing cytotoxic T cell development and culture in GM-CSF and IL-10 generates moDCs which render T cells anergic and drive the expansion of suppressor T cells (reviewed in (Ueno et al., 2010a)). Clearly moDCs differentiated in vivo will encounter a vastly more complex environment, and how these, let alone the steady-state developed DCs, compare to the in vitro generated DCs is not well characterized. Most studies on the cell biology of human DCs rely on GM-CSF and IL-4 generated moDCs, and for paper II we sought to expand our knowledge of established moDC responses to determine whether there was a transcriptional/translational rationale for Ii function in established endosomal dynamics. Both the transcriptional transient increase and decrease of CIITA and MHC II (but not Ii or the total MHC II population), the MIIC tubulation and MHC II cell surface increase have previously been shown for moDCs and these correspond to events in the conventional DC subsets, but not pDCs, in vivo. We therefore reasoned that moDCs were an acceptable experimental model in which to examine these events.

Relative quantitative Real-time PCR

In paper II we use quantitative real time polymerase chain reaction (qRT-PCR) to examine the relative ratios of MHC II and Ii isoform mRNA levels through DC maturation. As our moDCs were generated from monocytes isolated from several individuals with variable haplotypes, we needed primers which covered most allelic variants of the MHC II isotypes. Primers were consequently designed to target conserved loci on the MHC II alpha chain mRNAs which display less allelic diversity than the beta chains (Robinson et al., 2010). As Ii can form heterotrimers and associates with all MHC II isotypes

we assessed the total MHC II population (HLA-DR, HLA-DP and HLA-DQ) to determine the ratio of total MHC II to the total Ii (p33/35 and p41/43). All primers were extensively tested for specificity and efficiency by melting curve analysis and ten-fold serial dilutions ($E = 10^{-1/slope}$). For reference we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) due to its consistent, constant level in all donors examined. Another commonly used reference gene, β-actin, exhibited marked fluctuations after moDC activation, possibly due to the increased migratory capacity in this maturation state. SYBR®Green was used as fluorescent reporter to monitor amplification of specific targets and threshold cycle (Ct) values were corrected for primer efficiency according to equation:

$$R = \frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{reference})^{\Delta Ct\ reference\ (control-sample)}}$$

Relative qRT-PCR is, like microscopy above, fraught with potential for error in sample preparation, running and analysis. In our analysis we have consistently used the same protocols, reagents and instruments in our data-acquisition and the same normalization and analytical procedures in order to be able to compare our different data sets. We have to the extent possible tried to adhere to the most rigid experimental and analytical guidelines (Bustin et al., 2009). Working with moDCs generated from different individuals will necessarily include some deviation, however despite these premonitions our samples displayed a surprisingly low variation and consistent regulation in all donors examined.

An aspect which requires some consideration is the extent to which the respective mRNAs result from transcription or other mechanisms. The 3' and 5' ends of mRNAs are protected by the poly A tail and the 7-methyl-guanosine cap, respectively, and the removal of any of these initiates mRNA degradation by exonucleases. The target of the majority of mammalian micro-RNAs are located in the 3' UTR (un-translated region) of mRNAs, and adenine-uridine rich elements (AREs) located in the 3' ends can confer constitutive instability on mRNAs containing them (Anderson, 2010), no such elements have been identified in the MHC II or Ii transcripts, although CIITA is a specific target of miRNAs (Asirvatham et al., 2008). Despite this we cannot exclude that the mRNA species examined have variable half-lives through maturation. Translational regulation of several mRNA species has been shown in moDCs after activation with LPS, but neither the Ii or MHC II isotypes have been found to be differentially translated (Ceppi et al., 2009). However, the steady state affinity of these mRNAs for ribosomes, and the consequent translational activity is unknown, so a correlation between mRNA levels and translation products, beyond the crude biochemical assays presented in paper II, could not be established. Post-transcriptional mechanisms that modify mRNA stability and/or translation could consequently influence the levels of mRNA and the translation efficiency (for a review see (Anderson, 2010)).

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we have examined the role of Ii in modulating the endosomal system to promote an APC specific endocytic environment devoted to antigen presentation on MHC II. Our findings presented in paper I (Landsverk et al., 2010b) clearly show that Ii is involved in the generation of a slowly maturing endocytic pathway that is accessed not only by new, Ii-associated MHC II, but also mature MHC II. We show that this increases the half-life of

MHC II, but also of the epidermal growth factor (EGF) receptor which is sorted onto intralumenal vesicles after EGF stimulation. Placed in the context of the large body of research generated over the past three decades (as discussed in the theoretical introduction and (Landsverk et al., 2009)), our results strongly suggest that Ii plays a major role in the biogenesis of an alternate endocytic pathway devoted to MHC II peptide loading. This Ii induced pathway exhibits all the characteristics of the MIICs observed in B cells and steady state DCs, and allows for the accumulation of intracellular MHC II for exosome release or tubule mediated delivery of MHC II upon DC activation. In paper II we examine the molecular basis for a role for Ii in the endomembrane dynamics occurring during DC maturation and find that there is a sustained production of Ii in mature moDCs. This surplus of Ii is predominantly present in the ER, but a fraction traffics to the endocytic pathway despite the reduced availability of new MHC II. This provides a biosynthetic rationale for a role for Ii in modulating the endocytic pathway also in mature moDCs.

The multitude of Ii-interacting partners described has shown that this molecule is intimately involved in multiple aspects of antigen presentation and beyond. How many of these partners benefit from the endosome modulating capacity of Ii is poorly examined so far, but presumably CD1 molecules and MHC I could gain the same benefits as MHC II with regard to antigen loading. For MHC I, this suggests that Ii may play a role in the cross-presentation of exogenous antigens to CD8+ T cells. The delayed proteolysis of antigen and early endosomes are essential to achieve the loading of peptides on MHC I, and Ii might thereby facilitate both proteasome/TAP (Transporter associated with antigen processing) dependent and independent pathways for peptide loading of MHC I in endosomes (for reviews on cross-presentation see (Amigorena and Savina, 2010; Ramachandra et al., 2009)). In this context it is interesting to note that one of the major cross presenting DC subsets, the pDCs, maintain CIITA activity and new MHC II/Ii production after activation (Young et al., 2008). Signaling receptors, such as TLR4, can maintain signaling from early, but not late endosomes (Palsson-McDermott et al., 2009). It is possible that Ii-associated chemokine receptors might receive similar benefits from an expanded early endosomal phase provided by Ii (Landsverk et al., 2010b). Alternatively, as Ii associated MHC II is not a substrate for ubiquitin E3 ligases (van Niel et al., 2006), interactions with Ii might influence signaling by delaying receptor ubiquitination and degradation.

The function of Ii in intracellular trafficking and endosomal modulation in many ways resembles the role of small GTPases in membrane fusion and motility. Which factors Ii influences to achieve these profound effects on endosomes are so far unknown, but their identification will undoubtedly provide information, not only on the particulars of the APC specific endosomal system, but also on the general properties of the endocytic pathway common to all cell types. No SNAREs have so far been isolated with Ii, but the rapid processing of Ii in the endocytic pathway, implies that any such interaction would be fleeting and difficult to isolate. The association with myosin II is intriguing, and could possibly serve additional purposes apart from controlling convergence of BcR internalized antigen and DC motility. Actin has been shown to play multiple roles in the endocytic pathway (Girao et al., 2008), and myosin II mediated recruitment of actin to Ii endosomes might play some hitherto unknown function here. An intriguing aspect is how this

function curtailed in APCs. Enlarged endosomes are not usually present in DCs or B cells, and despite a high expression, Ii does not produce a visible enlarged morphology in human moDCs. In humans, ii p35 and Ii p43 contain an ER retention motif, and Ii trimers containing one or more of these exhibit impaired ER export in the absence of MHC II. In transfected cell lines lacking MHC II, the presence of Ii p35 seems to restrict the generation of enlarged endosomes by Ii p33 (Sand and Gregers, unpublished). However, as the ER retention motifs are absent in Ii isoforms from mice, other factors must also be involved. Potential factors could be caspases which cleave the cytoplasmic tail of Ii. A putative caspase cleavage site has been identified in the cytoplasmic tail of Ii (Huang et al., 2008), and cleavage in this motif (DQRD) would remove the negative charge required for the induction of enlarged endosomes (Nordeng et al., 2002) (se also figure 3 in introduction). Interestingly, this cleavage might be developmentally regulated. In mice, nitric-oxide Synthase 2 (NOS2) was co-immunoprecipitated with Ii from BMDCs and Mθs after LPS activation, but not from immature cells, and the proximal production of nitric oxide was proposed to inhibit caspase mediated cleavage of Ii (Huang et al., 2008). Alternatively, the morphological effect of Ii could be compensated for by the presence of other APC specific factors such as rabs and/or ubiquitin ligases. Retrieval of membrane (vesicles) from early endosomes could be achieved through elevated recycling through CIITA induced Rab4b (Krawczyk et al., 2007), Ii-myosin II recruitment of actin for recycling microdomains (Puthenveedu et al., 2010), or enhanced membrane invagination and intralumenal packing through APC specific ubiquitin ligases acting on MHC II.

To our knowledge Ii is the only factor not specifically devoted to endo-membrane dynamics which can exert such profound effects on the endocytic pathway. This function is remarkably conserved, Ii cloned from humans, mice (Gedde-Dahl et al., 1997), chicken (Bremnes et al., 2000), and even Salmo salar (McAdam and Gregers, unpublished) all maintain the capacity to generate enlarged endosomes. This strongly argues for an evolutionary drive to maintain this property, however despite a sustained effort by our lab over many years, and several publications, this function of Ii has received little notice in the immunological community. For the future it should be a goal to separate the primary function of Ii on MHC II trafficking from its secondary profound effects on the endosomal system, and determine its impact on the diversity of peptide-MHC II combinations presented to T cells. The tools to do this are at our disposal, and the future should provide conclusive evidence to verify or discard this property of Ii in MHC II antigen presentation.

In conclusion, although the three decades that have passed since the discovery of Ii have greatly increased our knowledge of the function of Ii in MHC II assembly and transport in the endocytic pathway, there still remain many unsolved questions, and additional roles are still being discovered for this versatile molecule. Further insight into these mechanisms might provide us with a better chance of manipulating them in order to find new approaches to combat cancers, autoimmunity and infectious diseases.

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PAPERS

PAPER I

Invariant chain increases the half-life of MHC II by delaying endosomal maturation

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PAPER II

Regulation of invariant chain through monocyte derived dendritic cell maturation

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II