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Mature dendritic cells use endocytic receptors to capture and present antigens

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Mature Dendritic Cells Use Endocytic Receptors to Capture and Present Antigens

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Abstract

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In response to inflammatory stimuli, dendritic cells (DCs) trigger maturation, a terminal differentiation program required to initiate T lymphocyte responses. A hallmark of maturation is downregulation of endocytosis, widely assumed to restrict the ability of mature DCs to capture and present antigens encountered after the initial stimulus. We found that mature DCs continue to internalize antigens, especially by receptor-mediated endocytosis and phagocytosis. These antigens were transported to lysosomal compartments, loaded onto MHCII, and presented efficiently to T cells, both *in vitro* and *in vivo*. Antigens were also presented on MHCI with high efficiency. While mature DCs down-regulate macropinocytosis, they capture antigens via endocytic receptors and, in principle, remain able to initiate immune responses during the course of an infection.

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List of Abbreviations

APC	Antigen presenting cell
APC	Allophycocyanin
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CHX	Cycloheximide
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
FITC	Fluorescein-5-isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
HRP	Horseradish peroxidase
IC	Immune complex
iDC	Immature DC
IL-2	Interleukin-2
ITPG	Isopropyl β-D-1-thiogalactopyranoside
i.p.	Intraperitoneal
i.v.	Intravenous
LAMP-2	Lysosome associated membrane protein-2

LB	Lysogeny broth
LN	Lymph node
mDC	Mature DC
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class II
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
TAP	Transporter associated with antigen processing
TLR	Toll-like receptor
TMB	3,3′,5,5′-tetramethylbenzidine

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Chapter 1: Introduction

The Immune Response

For as long as there have been multi-cellular organisms, there have been simpler organisms seeking niches within their nutrient-rich hosts (Pancer and Cooper, 2006). While there are many examples of mutualism, invasion often comes at a great cost to the host (Lefevre et al., 2009). The immune system is the collection of mechanisms that the host employs to detect, and remove pathogenic threats (Murphy et al., 2007).

The immune system can most broadly be divided into the arms of innate immunity and adaptive immunity (Murphy et al., 2007). The ancient innate immune system, present in some form among virtually all metazoan organisms, detects pathogens using genomically encoded receptors that recognize conserved microbial molecular patterns (Palm and Medzhitov, 2009; Takeda and Akira, 2007). Pathogens recognized as foreign by these receptors trigger an inflammatory response and can be eliminated though any of a variety of strategies. Phagocytosis, opsonization, lysis through complement cascade, toxic metabolites such as reactive oxygen species and antibacterial peptides are just a few of the available mechanisms available to the host (Murphy et al., 2007).

It was much more recently in evolutionary history (approximately 450 million years ago) that adaptive immunity provided hosts with the ability to engineer custom-made receptors for each newly encountered pathogen (Cooper

and Alder, 2006). All vertebrates with jaws possess such adaptive immune systems. The somatic recombination of gene segments within antigen-receptor loci leads to the generation of vast populations of B cells and T cells, each expressing a novel receptor (Bassing et al., 2002; Schatz, 2004). These individual cells are capable of amplification when, by chance, their receptors find their pathogenic match (Bartl et al., 1994). In addition to increased specificity, adaptive immunity supplies jawed vertebrates with the advantage of "immunologic memory." Once a custom designed receptor is amplified after exposure to a foreign threat, it becomes a part of the host's immunologic arsenal, and is ready to quickly act in the future. This greatly reduces the risk of future infections by the same pathogen (Gourley et al., 2004).

Dendritic Cells: Bridging Innate and Adaptive Immune Responses

Despite the fact that medicine had been taking advantage of adaptive immune responses for centuries through vaccinations (Plotkin, 2005), the way in which the majority of adaptive immune responses are initiated was determined only within the past few decades. In 1973 Ralph Steinman and Zanvil Cohn published the first report of a unique cell type in the spleen which they termed the "dendritic cell" because of its numerous fine extensions (Steinman and Cohn, 1973). Over the following years, studies revealed that they were more than 100-fold more potent at initiating immune responses than cells such as monocytes and macrophages, which had previously been thought to be chiefly responsible for

lymphocyte activation (Steinman and Witmer, 1978). However, a better understanding of how these cells initiate adaptive immune responses would have to wait until the interconnected nature of the innate and adaptive immune systems was unraveled.

When DCs were discovered, the innate immune system was considered to be primarily a rapid but incomplete means of controlling infection until the slower but more powerful adaptive immune system could be mobilized (Fearon and Locksley, 1996). This view was challenged by a seminal 1989 article, in which Charles Janeway hypothesized that the innate immune system played a vital role in initiating and directing adaptive immunity. His proposal was that the immune system distinguished pathogen derived-antigens from self-antigens by using a set of pattern recognition receptors (PRRs) that were capable of recognizing pathogen associated molecular patterns (PAMPs) (Janeway, 1989).

Janeway's hypothesis was validated in 1997 with the discovery of a mammalian homologue of the insect protein known as Toll (Medzhitov and Janeway, 1997). In *Drosophila*, in addition to regulating polarity during embryogenesis (Hashimoto et al., 1988), Toll participates in the innate response against fungal pathogens (Lemaitre et al., 1996). Interestingly, the mammalian Toll-like receptor (TLR) was shown to activate the NF-κB pathway, a key signaling pathway involved in a host of inflammatory responses (Medzhitov and Janeway, 1997). Subsequently, it was determined that it was a mutation in a member of the TLR family, TLR4, that accounted for the lack of response to the bacterial cell wall

component lipopolysaccharide (LPS) in two strains of mice that had been noted for this defect (Poltorak et al., 1998). Together, these discoveries demonstrated that the immune system recognizes microbial products to distinguish self from non-self.

It is now known that most mammalian species express between 10-15 distinct TLR members on the plasma membrane and within endocytic compartments of specialized cell types (Akira et al., 2006; Iwasaki and Medzhitov, 2004). These receptors recognize a broad array of pathogenic features including LPS, peptidoglycan, zymosan, flagellin, ssRNA, CpG DNA and profilin (Takeda and Akira, 2007). Such TLR ligands are similar in that they are not present in their metazoan hosts, thus making receptor ligation a signal of pathogenic threat. Just as importantly, the ligands are essential to the function of the pathogen, making it difficult for the forces of natural selection to significantly alter them to evade detection within the host. (It should be noted that this is now somewhat of an oversimplification since not all foreign species are pathogens (e.g. gut flora) and more recent studies have associated certain types of TLR signaling with host homeostasis (Lee et al., 2006; Rakoff-Nahoum et al., 2004)).

The PRRs of the innate immune system are now known to extend beyond the prototypical TLR family. TLRs are transmembrane proteins that initiate signaling from the extracellular space as well as from within endosomal compartments. It is becoming clear that lectins may play a similar role. Dectin-1, a C-type lectin, is similar to TLRs in that it initiates inflammatory signaling pathways (Rogers et al., 2005) but different in that it is also a endocytic receptor

(Herre et al., 2004). There are also a variety of cytosolic PRRs capable of detecting pathogens from within the topologically distinct intracellular space. These receptors include Nod/Nod2, RIG-1, and the ISD sensor which recognize peptidoglycans, cytosolic viral RNA, and cytosolic viral DNA respectively (Palm and Medzhitov, 2009).

In most tissue types, signaling induced by any of the panopoly of innate receptors of can lead to the recruitment of a rapid innate response. This is mediated by cell types such as neutrophils, monocytes, NK cells, eosinophils and basophils through the secretion of inflammatory cytokines, chemokines, and anti-microbial peptides (Iwasaki and Medzhitov, 2004). DCs are unique, however, in that they do not merely contribute to this inflammatory response but initiate the adaptive response. PAMP recognition at the site of an infection is frequently accompanied by the internalization of pathogen-derived protein antigens by DCs. Through the processing of these antigens and the subsequent presentation of pathogen-derived peptides, DCs instruct the adaptive immune system on the identity of the threat, while using co-stimulatory molecules and cytokines to induce antigen-specific T cell expansion (Reis e Sousa, 2004; Trombetta and Mellman, 2005).

The Maturation Paradigm

In response to TLR activation, DCs undergo a dramatic set of functional and morphological changes, termed maturation, that rapidly occur after contact with an

inflammatory stimulus (Trombetta and Mellman, 2005). Early evidence that DCs did not maintain a stable phenotype included the observation that while DCs greatly increased their ability to stimulate allogenic T cells after culture for 24 hours, only freshly prepared cells could efficiently process and present specific antigens to T cell clones (Romani et al., 1989). It soon became clear that the increased ability to present antigen was largely due to a rapid and dramatic reorganization of cellular function.

This cellular reorganization allows DCs to transition from specializing in antigen acquisition to specializing in antigen presentation (Reis e Sousa, 2006). Among the first changes that occurs is a rapid increase in macropinocytosis, followed by a nearly complete down-regulation (Garrett et al., 2000; West et al., 2004). Newly stimulated DCs are in a potentially antigen-rich environment, and it has been suggested that increased antigen uptake would maximize the potential of initiating an immune response against the foreign target that activated the DC in the first place. The rapid down-regulation of macropinocytosis that follows has been used to support evidence for the so-called "snapshot hypothesis". This is the idea that because a DC ceases to take up new antigen after the initial stimulus, it presents a "snapshot" of the antigenic environment hours, or even days later (Ingulli et al., 1997; Villadangos et al., 2005). It has also been proposed that this prevents mature DCs from taking up self-antigens after activation, thus acting as a safeguard against potential autoimmunity (Thery and Amigorena, 2001).

Many other cellular changes occur as well. Essential to the maturing DCs cellular transformation is the reorganization of MHCII from the late endocytic compartments to the plasma membrane (Cella et al., 1997; Pierre et al., 1997). Activation leads to an initial burst in MHCII synthesis (Cella et al., 1997) and the formation of tubulo-vesicular structures highly enriched with peptide-MHC II complexes destined for the plasma membrane (Boes et al., 2002; Chow et al., 2002). Another specialization leading to optimal antigen processing includes acidification of the lysosomal compartment leading to enhanced proteolysis (Trombetta et al., 2003). While peptide-MHCII complexes that reach the cell surface are rapidly internalized in immature DCs, these structures are stabilized on the surface of mature DCs due to the loss of ubiquitination of MHCII (Shin et al., 2006; van Niel et al., 2006). This leads to a 10-fold increase in MHCII half-life and increases the likelihood of peptide-MHC complex recognition by T cells (Cella et al., 1997).

Migration may or may not accompany DC maturation (Reis e Sousa, 2006). Both the spleen and lymph nodes contain populations of resident DCs that are in close proximity to T cells (Villadangos and Heath, 2005). However, peripheral DCs are stationed at sites including the skin, gut, lungs: the interface between the host and the external environment (Iwasaki, 2007). While their localization is critical to ensuring DCs detect pathogens early in the course of an infection, such positioning often leaves them a great distance from the T cells in the draining lymph nodes and spleen where they must traffic (Alvarez et al., 2008; Ingulli et al.,

1997; Randolph et al., 2005). Thus, the maturation program includes up-regulation of proteolytic enzymes such as MMP-2 and MMP-9 that are necessary for migration through extracellular matrix-rich tissues and up-regulation of CCR7, a key chemokine receptor responsible for DC tropism for the LN (Bonasio and von Andrian, 2006)

Antigen Capture

All eukaryotic cells demonstrate at least some form of endocytosis as internalization and recycling of plasma membrane components is essential to cellular homeostasis (Mellman, 1996). Specialized cell types within complex multicellular organisms use various forms of endocytosis to control a sweeping range of activities from neuronal transmission to host defense (Mellman, 1996). DCs are specialized in this latter function, as the first step in initiating an immune response is antigen capture (Trombetta and Mellman, 2005).

DCs acquire both self and foreign antigens through one of several forms of endocytosis, most broadly divided into phagocytosis, the uptake of large particles (>0.5μm), and pinocytosis, the uptake of fluids or solutes (Conner and Schmid, 2003). While phagocytosis is only carried out by specialized cell types, DCs being among them, pinocytosis occurs in all cell types by one of several possible mechanisms. These include clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolae-independent endocytosis, and macropinocytosis (Conner and Schmid, 2003). Of these mechanisms,

phagocytosis, clathrin-mediated endocytosis and macropinocytosis have all been extensively examined in DCs and have been implicated in antigen presentation (Garrett et al., 2000; Sallusto et al., 1995; Savina and Amigorena, 2007).

Antigen Capture: Phagocytosis

> Whenever the organism enjoys immunity, the introduction of infectious microbes is followed by the accumulation of mobile cells, of white corpuscles of the blood in particular which absorb the microbes and destroy them. The white corpuscles and the other cells capable of doing this have been designated 'phagocytes,' (i.e., devouring cells) and the whole function that ensures immunity has been given the name of 'phagocytosis' (Metchnikoff, 1908).

These were the words of the Russian embryologist Ilya Metchnikoff as he accepted his Nobel Prize along with Paul Ehrlich in 1908. Years earlier, after observing what appeared to be ingested cells within sea star larvae, he successfully demonstrated these cells were capable of recognizing and internalizing foreign objects. He found that similar phagocytes existed in humans and began accumulating evidence that they played an important role in host defense (Stuart and Ezekowitz, 2005). His view proved to be remarkably prescient as specialized cells such as DCs, neutrophils, monocytes and macrophages employ phagocytosis when clearing pathogens and apoptotic cells or when remodeling tissue (Conner and Schmid, 2003).

Phagocytosis typically proceeds after a specialized cell recognizes an appropriate ligand on the surface of a particle. Target recognition is possible

through one of two strategies. The first is through direct binding of a ligand by specific receptors. Examples include recognition of phosphatidyl serine on apoptotic cells by TIM-4 (Kobayashi et al., 2007; Miyanishi et al., 2007) or recognition of bacterial carbohydrates by C-type lectins (Figdor et al., 2002). The second strategy is through recognition of soluble pattern recognition receptors that have opsonized the target. This strategy, which effectively increases receptor diversity, includes recognition of IgG by the FcγR or complement by the complement receptor (Stuart and Ezekowitz, 2005).

Interestingly, the mechanism of phagocytosis differs depending on the receptors that are engaged (Kerrigan and Brown, 2009). FcγR mediated phagocytosis of an opsonized particle engages a signaling cascade involving the small GTPases Rac1, Cdc42 and Rho (Cox et al., 1997; Garrett et al., 2000; Massol et al., 1998). This leads to an actin-dependent advance of plasma membrane over the surface of the particle, leading to further engagement of FcγRs and the continuing engulfment of the particle (Greenberg et al., 1990; Swanson, 2008). In contrast, the phagocytosis of complement-opsonized particles requires Rho but not Cdc42 and Rac1 (Caron and Hall, 1998). The actin-rich pseudopodia seen during FcγR-mediated phagocytosis particles are notably absent, and the resulting phagosome is also more spacious (Kerrigan and Brown, 2009).

Through a series of fusion and maturation events, the newly formed organelle encasing the internalized particle progresses functionally and semantically from phagosome to phagolysosome (Garin et al., 2001). Especially in

macrophages, this compartment is specialized for the killing of pathogens (Stuart and Ezekowitz, 2005). Reactive oxygen species, formed by NADPH oxidase, participate in both the direct killing of pathogens and the activation of proteases which also contribute to pathogen destruction (Reeves et al., 2002).

DCs use this same machinery to increase the efficiency of cross-presentation antigens on MHCI. The NADPH oxidase NOX2 is recruited to the phagosome, producing low levels of reactive oxygen species. While these levels are not optimal for pathogen killing, they lead to a sustained alkalinization of the phagosomal compartment. This leads to a less degradative environment, which is optimal for the formation MHCI-peptide complexes (Savina et al., 2006).

DCs, now considered a quintessential phagocyte, were initially described as non-phagocytic (Steinman and Cohn, 1974). In fact what was observed was the modulation of DC phagocytosis that occurs after DC maturation. Freshly isolated cells were highly phagocytic, but lost this ability when put in culture (Reis e Sousa et al., 1993). Down-regulation of phagocytosis can also be induced after the introduction of inflammatory stimuli such as LPS or CpG DNA, both *in vitro* and *in vivo* (Garrett et al., 2000; Wilson et al., 2006).

Antigen Capture: Macropinocytosis

Macropinocytosis involves the uptake of large volumes of extracellular fluid by ruffles of plasma membrane extended via localized actin filament assembly (Swanson, 2008). While different from phagocytosis in that uptake is not directly

triggered by a particular ligand, the cellular machinery and regulation of macropinocytosis are otherwise quite similar. Despite the fact that there is relatively little evidence of its relevance *in vivo*, this form of antigen acquisition has been studied and modeled extensively in DCs (Sallusto et al., 1995; Steinman and Swanson, 1995).

There are several reasons for this. First, while many cell types can be induced to perform macropinocytosis by the addition of specific factors such as PDGF or EGF, or by pathogens, initial studies showed that DCs are constitutively macropinocytic (Sallusto et al., 1995). Thus, it seems intuitive that DCs would use this method of antigen uptake to widely sample their environment under steady state conditions.

Second, these cells are capable of taking up a volume of extracellular fluid comparable to the volume of the cell itself in approximately one hour (Sallusto et al., 1995). Because of the impressive endocytic rate and non-specificity, macropinocytosis is the most easily studied endocytic route *in vitro*. Antigen or endocytic tracers can be internalized by DCs in high quantities, simply by adding these reagents to tissue culture medium.

Lastly, and perhaps most importantly, the macropinocytic rate is modulated by pathogen presence and inflammatory mediators (Falcone et al., 2006; Garrett et al., 2000; West et al., 2004). Immediately after DC activation with a variety of TLR stimuli, there is a transient burst of endocytic activity (West et al., 2004) followed by down-regulation, mediated at least in part by a reduction of the active form of

the Rho GTPase, Cdc42. (Garrett et al., 2000; Sallusto et al., 1995). Thus, the cell biology of DC macropinocytosis could be fit immediately into an immunological context: these cells efficiently take up antigen when they first encounter a pathogen, but then cease further antigen uptake, perhaps preventing autoimmunity (Reis e Sousa, 2006).

Antigen Capture: Receptor/Clathrin Mediated Endocytosis

All mammalian cells continuously use endocytic receptors, internalized via clathrin-coated pits, to recruit essential nutrients such as low-density lipoproteins and transferrin (Anderson et al., 1982; Dautry-Varsat et al., 1983). Specialized leukocytes such as DCs, macrophages and neutrophils use this pathway to internalize endocytic receptors along with their potentially antigen-rich ligands. There are increasing numbers of receptors that have been identified as physiologically relevant for the uptake of foreign and self-antigens via clathrin coated pits (Kerrigan and Brown, 2009).

The FcγR and the mannose receptor were among the first endocytic receptors characterized in DCs (Steinman, 1991). The FcγR was shown to mediate the efficient uptake of antibody/antigen complexes (immune complexes), a physiologically relevant source of potential antigens (Esposito-Farese et al., 1995). Antigens internalized as immune complexes are presented with high efficiency on both MHCI and MHCII (Amigorena and Bonnerot, 1999; Regnault et al., 1999). The mannose receptor has been shown to be at least partially responsible for

uptake and presentation of model antigens such as OVA and HRP (Sallusto et al., 1995; Tan et al., 1997). This receptor, a C-type lectin, is thought to be physiologically relevant for binding soluble self-glycoproteins as well as a variety of bacteria, viruses and fungi through recognition of pathogen-associated high mannose (Engering et al., 1997; Gazi and Martinez-Pomares, 2009; Sallusto et al., 1995).

The mannose receptor is part of a much larger family of C-type lectins, the largest family of endocytic receptors that has been characterized in DCs (Cambi and Figdor, 2005). Members of this family contain a highly conserved carbohydrate recognition domain (CRD) with a calcium binding pocket (Figdor et al., 2002). However, both the CRD and calcium-binding pocket have been shown to be non-functional in certain receptors that otherwise have high homology with other C-type lectin family members. In some cases these receptors have been shown to bind proteins or lipids (Kogelberg and Feizi, 2001).

DEC-205 is a particularly well-characterized C-type lectin. Its subcellular trafficking to the lysosomal compartment and recycling to the plasma membrane is well established (Jiang et al., 1995; Mahnke et al., 2000). It has also been studied extensively *in vivo*. Model antigens conjugated to anti-DEC-205 antibodies have been used to show that antigens introduced in the absence of other stimuli lead to T cell unresponsiveness and deletion (Bonifaz et al., 2002; Hawiger et al., 2001). Still other studies have shown how similar antibodies can efficiently mediates presentation of HIV epitopes (Bozzacco et al., 2007). Despite this extensive

characterization and modeling as a potential vaccine target, its physiologic role is poorly understood. This may change as a recent study has indicated that it binds and facilitates the uptake of apoptotic and necrotic cells (Shrimpton et al., 2009).

DCs express a variety of other C-type lectins as well. DC-SIGN has been noted for its ability to bind HIV, though like the mannose receptor it has also been shown to bind mannan (Engering et al., 2002b). Dectin-1 has been shown to bind β glucans, a component of fungal cell walls and the receptor can mediate uptake of a series of fungal species including *C. albicans*, *S. cerevisiae* and A. *fumigatus* (Herre et al., 2004). There are still a number of lectins such as Dectin-2 and DCIR that have no known physiologic ligands.

A feature that these receptors share is that they are all internalized via clathrin-coated pits. Clathrin-mediated endocytosis involves the assembly of clathrin, adaptor and accessory proteins along the inner leaflet of the plasma membrane associated with the cytosolic domains of aggregated surface receptors and their ligands (Conner and Schmid, 2003). Receptors are aggregated into coated pits at concentrations approximately two orders of magnitude greater than in the rest of the plasma membrane (Pearse and Robinson, 1990). These networks of clathrin and accessory proteins lead to membrane bending and budding. The ultimate fission step involves the GTPase dynamin, which constricts the vesicle neck though the formation of a collar of interconnected dynamin rings (Hinshaw and Schmid, 1995). Additional forces may be provided by myosin and actin (Ungewickell and Hinrichsen, 2007).

After release into the cytoplasm, the newly internalized vesicles lose their coats, allowing them to fuse with the early endosome compartment. This compartment, a dynamic collection of vesicles and tubules, has been considered a location where cargo destined for degradation is separated from cargo destined for recycling (Mellman and Warren, 2000; Schmid et al., 1988; Seaman, 2008). Newer data suggests that the sorting event may actually occur during internalization as different receptors sort to distinct early endosomal compartments (Lakadamyali et al., 2006).

Antigen Processing

As outlined earlier, DCs initiate immune responses by presenting antigen to, and thus activating, naïve T cells (Banchereau and Steinman, 1998). However, the TCR, whose activation is the gateway to the entire array of effectors within the adaptive immune response, cannot recognize unprocessed antigens (Davis et al., 1998). Activation occurs when small antigen-derived peptides are loaded onto MHC molecules within the organelles of an antigen-presenting cell and these complexes are trafficked to the plasma membrane to be made available for recognition (Davis et al., 1998; Trombetta and Mellman, 2005).

There are two main classes of MHC molecules, MHCI and MHCII, which define the type of T cells that are activated. Virtually all cells are capable of loading antigens onto MHCI thus activating CD8+ T cells (though DCs are uniquely able to prime naïve CD8 T cells). However, DCs are one of only a small group of

"professional antigen-presenting cells" which express MHCII and can therefore present antigens to CD4+ T cells (Trombetta and Mellman, 2005).

The topological origin of an antigen broadly determines whether an antigen will be presented on MHCI or MHCII, and therefore the immune response that will be generated against this antigen (Trombetta and Mellman, 2005). While there are many exceptions to this rule, some of which will be discussed, intracellular antigens are classically loaded on MHCI while extracellular antigens are presented on MHCII (Ackerman and Cresswell, 2004; Trombetta and Mellman, 2005; Wilson and Villadangos, 2005)

The majority of antigens that are endocytosed are trafficked to the lysosomal compartment. In most cell types this is a terminal, degradative compartment and any potential antigenic epitopes would be lost. Rather DCs have modified MHCII rich lysosomes that are optimized for antigen loading (Chow and Mellman, 2005; Kleijmeer et al., 1997). Instead of hydrolyzing proteins to individual amino acids, DCs relatively mild lysosomes generate peptides for loading on MHCII (Delamarre et al., 2005).

MHCII follows its own path to the lysosomes after it is synthesized on rough ER so it can bind newly generated antigenic peptides. Newly synthesized MHCII is folded in the ER along with the chaperone, invariant chain (Cresswell, 1994; Trombetta and Mellman, 2005). The invariant chain has at least two main tasks. The first is trafficking MHCII to the endocytic compartment, where it can encounter antigen. The second function is performed by an invariant chain fragment called

CLIP. It sits in the peptide-binding groove where it protects MHCII from pre-mature loading of antigen (Bakke and Dobberstein, 1990; Cresswell, 1996). Peptide-loading occurs with the assistance of HLA-DM, a MHC-like molecule that catalyzes the removal of CLIP from the peptide-binding groove (Denzin and Cresswell, 1995). Once loaded with antigen, MHCII is trafficked to the plasma membrane in tubulovesicular structures (Boes et al., 2002; Chow et al., 2002). In immature DCs, MHCII is ubiquitinated and is rapidly internalized from the cell surface. In mature DCs, lack of ubiquitination leads to the stabilization of MHCII on the cell surface (Shin et al., 2006; van Niel et al., 2006).

DCs are not unique in their expression of MHCI, which is present on the cell surface of virtually all nucleated cell types. However, presentation of antigens on MHCI by DCs is vital since they appear necessary to prime naive CD8+ T cells for the generation of a cytotoxic T cell response (Jung et al., 2002). The MHCI pathway is considered primarily a pathway of endogenous antigen presentation because the pathway originates in the cytosol (Pamer and Cresswell, 1998). Cytosolic proteins are fragmented by the proteasome and transported via TAP into the lumen of the ER where stable MHCI/peptide complexes are formed with the assistance of the chaperone tapasin, calreticulin and ERp57 (Peaper and Cresswell, 2008).

The topology of this antigen-processing pathway favors cytosolic proteins, which have access to the proteasome and TAP. However, because DCs must also induce immune responses against antigens that do not directly infect them, DCs

can also prime responses against exogenous antigens through a process called cross-presentation. Internalized antigens are introduced to the MHCI processing pathway (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1995). Many mechanisms have been proposed including ER-phagosome fusion (Ackerman et al., 2006; Guermonprez et al., 2003), transit of soluble antigen to the ER (Ackerman et al., 2005), loading of MHCI recycled from the cell surface (Gromme et al., 1999), various mechanisms of increased endosomal permeability (Reis e Sousa and Germain, 1995; Rodriguez et al., 1999), and even direct introduction through gap junctions (Neijssen et al., 2005). While many of these mechanisms may play a role under certain circumstances, many details of this pathway require further definition.

The Route of Endocytosis Influences Antigen Presentation

It is becoming clear that the relative efficiency of cross-presentation on MHCI versus direct presentation on MHCII can be determined in part by the endocytic receptor that internalizes the antigen. Long before the implications for antigen processing and presentation were known, it was clear that functionally distinct populations of endosomes existed and were involved in trafficking internalized cargo to specific intracellular sites (Hewlett et al., 1994; Schmid et al., 1988). More recently it has been shown that distinct adaptors are involved in targeting different types of endocytic cargo (Robinson, 2004) and that sorting to

different populations of early endosomes occurs before the endosomes have even fully formed (Lakadamyali et al., 2006).

There are a variety of different trafficking patterns revealed after the internalization of DC endocytic receptors. Like the contents of macropinosomes, the FcγR is delivered to the lysosomal compartment where it is degraded (Mellman et al., 1983; Sallusto et al., 1995). In contrast, DEC-205 and the mannose receptor are recycled. However, while the mannose receptor recycles directly from early endosomes (Burgdorf et al., 2007; Mahnke et al., 2000), DEC-205 delivers antigen deep into the late endosomal and lysosomal compartments before recycling (Jiang et al., 1995; Mahnke et al., 2000). Another C-type lecin, DC-SIGN localizes to the lysosomal compartment after internalization in immature DCs but stays in the early endosomal compartment in mature DCs (Engering et al., 2002b).

We now know that this differential localization of cargo can bias antigen processing and presentation preferentially towards MHCI or MHCII pathways (Burgdorf and Kurts, 2008). Antigen targeted to late endosomes, either through macropinocytosis, or through late endosome-targeted liposomes is more efficiently presented on MHCII (Belizaire and Unanue, 2009; Burgdorf et al., 2007). In contrast, antigen targeted to early endosomes by the mannose receptor, transferrin receptor or early endosome-targeted liposomes is most efficiently targeted on MHCI (Belizaire and Unanue, 2009; Burgdorf et al., 2007; Burgdorf et al., 2008). It is no surprise that antigens delivered to an MHCII loading compartment would be better presented on MHCII than antigens that stayed localized to early endosomes.

However, the precise mechanism of how localization to early endosomes is beneficial to MHCI presentation is still poorly defined, though loading of MHCI in the endosomal compartment has been reported (Burgdorf et al., 2008).

DC Subsets

DCs have often been discussed as if they are one specialized cell type (as they have been thus far). However, they are actually a heterogeneous group. There are at least six main populations of DCs that can be found in lymphatic tissues that can be distinguished by the pattern of various surface markers that they express (Vremec and Shortman, 1997). DCs of the lungs, skin, gut, and other mucosal surfaces also differ from one another in surface marker expression and phenotype. While these subsets all present peptide antigens to T cells on MHCI and MHCII, it is becoming clear that they play different roles and preferentially initiate different types of immune responses (Heath et al., 2004).

One DC subset, the plasmacytoid DC differs from the remaining subtypes to such an extent that it has earned its own subgroup, with the remaining DC subsets collectively termed "conventional DCs" (Villadangos and Young, 2008). Plasmacytoid DCs produce exceptionally high amounts of type I interferon upon recognizing viral pathogens (Colonna et al., 2004). However, they are relatively poor at taking up exogenous antigens (Villadangos and Young, 2008). While these cells play a vital role in the initiation of both innate and adaptive antiviral

responses (McKenna et al., 2005), the scope of this dissertation is concerned chiefly with the subsets grouped as "conventional DCs."

Conventional DCs found in secondary lymphoid tissues can most broadly be divided into migratory DCs and resident DCs. Migratory DCs reside in peripheral tissues under steady state conditions and travel through lymphatics to reach the local draining lymph nodes where they are able to present peripheral antigens (Randolph et al., 2005). Migration can be induced when these DCs come into contact with inflammatory stimuli, though migration also occurs under steady state conditions (Wilson et al., 2003). In contrast, resident DCs reside in the LNs and spleen without having resided in peripheral tissues (Wilson et al., 2003).

The functional importance of having DCs present in spatially distinct locations has been explored. One implication is that there are two waves of antigen presentation when an inflammatory response is initiated in the periphery. The first wave is provided by resident DCs as they sample antigens draining from the periphery through the afferent lymphatics. The second wave is supplied by migratory DCs when they arrive in the LN up to 24 hours later (Itano et al., 2003). Other studies have shown that resident DCs can present antigens that are transferred to them directly by migratory DCs (Allan et al., 2003). The mechanism of this transfer has not been determined (Carbone et al., 2004).

Surface expression of CD8αα homodimer has been helpful in the further classification of murine DC subsets. While this surface marker has no known function in DCs, it has been used to separate resident splenic DCs into two main

groups, CD8α+ DCs and CD8α- DCs (Shortman and Liu, 2002). CD8α+ DCs are superior at cross-presentation and the presentation of cellular antigens on both MHCI and MHCII. (den Haan et al., 2000; Heath et al., 2004).

Initial studies suggested that this was due to the superior phagocytic abilities of CD8α+ DCs (Schulz and Reis e Sousa, 2002) or an increased ability to internalize dying cells (Iyoda et al., 2002). However, more recent work has suggested that antigen capture alone cannot explain the difference (Schnorrer et al., 2006). Gene expression profiles show that CD8α+ DCs are skewed towards the expression of proteins required for MHCI presentation such as TAP, ERAAP, tapasin and ERp57. CD8α- DCs in turn, are skewed towards expressing cathepsins, GILT, and H2-DM, all essential for MHCII presentation (Dudziak et al., 2007). These subsets also differ in the expression of endocytic receptors, such as DEC-205 and DCIR2(Dudziak et al., 2007). Differential expression of these and other receptors may provide DCs with both specificity in the pathogens they internalize as well the optimal trafficking of antigens to ideal processing compartments.

Summary

DCs play a fundamental role in initiating and directing adaptive immune responses. They widely sample their environment using several methods of endocytosis, then process and present peptide antigens to T cells. DC maturation, induced by pathogenic or endogenous stimuli, is the dramatic set of functional and

morphologic changes that allows these cells to present antigens in the optimal co-stimulatory context.

Can mature DCs acquire and present new antigens?

Largely because macropinocytosis and phagocytosis are greatly diminished in mature cells *in vitro* (Garrett et al., 2000; Reis e Sousa et al., 1993; Sallusto et al., 1995; West et al., 2004), the idea that immature DCs accumulate antigen while mature DCs present antigen is uniformly accepted (Banchereau and Steinman, 1998; Reis e Sousa, 2006). The idea has often been invoked to explain how DCs present pathogens acquired at the time of activation, while avoiding presentation of self-antigens encountered prior to or following pathogen encounter (Reis e Sousa, 2006; Thery and Amigorena, 2001) .

However, the cell biology of DCs suggests that the regulation of antigen capture and presentation may be more complex. First, mature DCs continue to form clathrin-coated vesicles (Garrett et al., 2000), suggesting that the internalization of selected surface molecules may still occur. Indeed, the internalization of MHCII ceases in mature DCs not because of any universal down-regulation of clathrin-mediated endocytosis, but because of a down-regulation of MHCII specific ubiquitin targeting. An artificial restoration of ubiquitination of the MHCII β chain leads to efficient internalization (Shin et al., 2006). In addition, mature DCs have been reported to internalize immune complexes *in vitro,* though the implications for presentation on MHCII was not assessed (Gil-Torregrosa et al., 2004). Together these findings indicate that receptor-mediated endocytosis of antigen by mature DCs was in need of direct analysis.

There have been occasional reports of mature DCs presenting newly acquired antigens in the literature. One study showed that mature Langerhans cells can acquire and present new antigens *in vivo* but not *in vitro* (Ruedl et al., 2001). Another study showed that DCs matured by CD40 ligation present antigen when matured by a particular stimulus (Nayak et al., 2006). However, they have failed to address the mechanism of presentation, or the substantial body of literature that supports the contrary model. These reports remain unconfirmed.

Here we formally test the widely accepted premise that mature DCs are unable to acquire and present new antigens. Most studies of endocytosis in DCs have involved exposing cells to high concentration of antigens or endocytic tracers taken up primariy by macropinocytosis. While it is clear that mature DCs lose the ability to present such antigens (Garrett et al., 2000), it is unclear whether antigen could be efficiently internalized by endocytic receptors, and if so, whether the antigen could then be processed and presented.

We therefore characterized the ability of mature DCs to internalize and present antigens using Fcγ receptors (FcγR) and the decalectin DEC-205 as model endocytic receptors, each having been documented to enable presentation on MHCI and MHCII (Amigorena and Bonnerot, 1999; Jiang et al., 1995; Regnault et al., 1999).

Specific Aims of This Study

1) Characterize the ability of mature DCs to capture and internalize antigens targeted to the DEC-205 and FcγR.

2) Determine the subcellular trafficking of antigens targeted to DEC-205 and the FcγR.

3) Determine whether receptor-targeted antigens are presented to T cells, both *in vitro* and *in vivo*.

Chapter 2: Internalization and Trafficking of Antigen by the FcγR and DEC-205 in Mature DCs

Introduction

We first set out to determine whether mature DCs continue to internalize endocytic receptors. While the modulation of macropinocytosis is controlled by RhoGTPases (Nobes and Marsh, 2000), there has been no similar mechanism described to explain how uptake via endocytic receptor might also be down-regulated. Instead, reduced surface expression of receptors has been proposed as a mechanism to prevent antigen uptake by mature DCs (Thery and Amigorena, 2001) though this has never been rigorously examined. We therefore decided to closely examine the surface expression and endocytic function of two well-characterized endocytic receptors, DEC-205 and the FcγR.

We first chose to examine the FcγR because of it's known physiologic importance (Guyre et al., 1997) and well characterized ability to present antigens derived from internalized immune complexes on both MHC I and MHC II (Machy et al., 2000; Regnault et al., 1999). Unlike other receptors of the immune system, which recognize conserved features of pathogens, or lipids associated with cell death, FcRs are rather unique in that they bind antibodies, which are in turn capable of binding virtually any antigen.

FcRs are composed of extracellular alpha chains that determine specificity for a particular class of Ig, and cytosolic signal tranduction subunits that determine

downstream signaling (Amigorena and Bonnerot, 1999). These signaling units are known as ITAMs and ITIMS, and are capable of initiating activating or inhibitory signals respectively. They are nearly identical to the signaling motifs associated with activation of B cell receptors and T cell receptors. The activating signals associated with ITAMs are also associated with internalization, vital for uptake and presentation of immune complexes by DCs (Daeron, 1997).

FcγRs can be broadly classified into two groups: high and low affinity. Only high affinity FcγRs are able to bind monomeric Ig. Low affinity FcγRs bind monomeric Ig with negligible affinity, yet efficiently bind multivalent complexes (Hulett and Hogarth, 1994). It should be noted that both high and low affinity FcγRs must be aggregated to be activated. To this end, monomeric Ig binds high affinity receptors first, which are then complexed by antigen. Low affinity receptors, however, must bind antibody-antigen complexes that have been pre-formed. In our study, by using immune complexes to probe endocytic function of FcγRs, we would be primarily probing the function of FcγRIII as the receptor is low affinity. Notably, since FcγRIII also has an ITAM, it is capable of endocytosis (Daeron, 1997).

The other receptor that we chose to examine was DEC-205. This endocytic receptor is a member of the mannose receptor family, and is expressed on Langerhans cells, BMDCs and CD8α+ splenic DCs. DEC-205 contains ten external, contiguous, C-type lectin domains (Inaba et al., 1995). Interestingly, none of these ten domains contain known amino acid sequences thought to be necessary

for binding carbohydrates (Figdor et al., 2002). No natural ligand had been suggested in the literature until recently.

While the first evidence was reported in 2003 (Small and Kraal, 2003), a more recent report supports the hypothesis that DEC-205 binds proteins associated with apoptotic and necrotic cells (Shrimpton et al., 2009). The authors of this report used a panel of IgG fusion proteins that included the various extracellular domains of the DEC-205 receptor. This panel was used to probe for potential DEC-205 ligands. Their results suggested that C-type lectin domains 3&4 and 9&10, bind ligands expressed by cells only after they were subjected to insults that led to apoptosis or necrosis. Thus, there is evidence accumulating that the physiologic role for DEC-205 is the uptake of self-antigen. Whether this leads to tolerance or immunity depends on the associated inflammatory signals (Hawiger et al., 2001; Shrimpton et al., 2009).

The cell biological fate of antigens bound by DEC-205 as well as the immune responses directed against such antigens have been determined by using monoclonal antibodies as surrogate ligands (Bonifaz et al., 2002; Hawiger et al., 2001; Mahnke et al., 2000). Antigen endocytosed *via* DEC-205 is trafficked to late endosomes and lysosomes. The receptor is then rapidly recycled back to the plasma membrane where it is available for further rounds of antigen capture (Mahnke et al., 2000). Internalized antigen is processed for efficient presentation on both MHC class II and MHC class I molecules (Bonifaz et al., 2002; Jiang et al., 1995).

Whether or not the FcγR, DEC-205 or other endocytic receptors continue to function in mature DCs has not been closely examined. Down-regulation of both macropinocytosis and surface expression of endocytic receptors have been identified as a major means of restricting antigen presentation to antigens encountered by immature DCs (Reis e Sousa, 2006; Thery and Amigorena, 2001). However, in most cells types studied, DEC-205 expression has actually been reported to be higher in mature DCs than in immature DCs (Butler et al., 2007; Engering et al., 2002a). One study of human monocyte derived DCs showed that DEC-205 was not internalized in mature DCs (Butler et al., 2007). However, no mechanism was reported to account for this, and this result has not been reported in other types of DCs.

There has also been fairly little work on immune complex internalization by mature DCs. FcγR expression is lower in mature DCs than immature DCs (Regnault et al., 1999; Sallusto et al., 1995), though the implications for immune complex internalization are not known. While immune complex internalization by mature cells has been documented, presentation of antigen on MHCII has not been examined (Gil-Torregrosa et al., 2004).

Unlike receptor-mediated endocytosis, the modulation of phagocytosis with maturation is well documented. Most reports have shown that phagocytosis is significantly down-regulated in mature DCs. In fact, DCs were initially shown to be non-phagocytic (Steinman and Cohn, 1974). It was later determined that these cells were proficient phagocytes when freshly harvested but that the cells down-

regulated phagocytosis when put into culture (Reis e Sousa et al., 1993). Cells matured by this method were unable to take up neither opsonized nor unopsonized targets (Steinman and Swanson, 1995).

Interestingly, in studies that examined phagocytosis after the application of inflammatory stimuli, the results are less clear-cut. DCs matured by LPS were shown to down-regulate uptake of bacteria (Garrett et al., 2000). Another study showed that LPS, CpG DNA and poly I:C all lead to substantial down-regulation of latex bead uptake *in vivo* (Wilson et al., 2006). However, more recently it was shown that neither CD40 nor LPS inhibited uptake of iron oxide particles *in vitro* (Nayak et al., 2006). We therefore decided that it would be appropriate to confirm that inflammatory stimuli inhibit phagocytosis in our system and if so, to determine whether signaling induced by FcγR ligation could overcome such blockade.

Results

Surface Expression of FcγR and DEC-205 on Immature and Mature DCs

First, we confirmed that the FcγR and DEC-205 were present on mature DCs. As we ultimately intended to use both BMDCs as well as DCs derived from lymphatic tissue, we tested DCs from these various sources after maturation with LPS. Studies of DCs in culture systems show that DCs complete the maturation program after approximately 20 hours (Garrett et al., 2000; Mellman et al., 1998). Studies looking at maturation *in vivo* have been complicated by the disappearance of DCs and by the reappearance of immature DCs at later time points (De Smedt et al., 1996). Therefore, recent studies assessing the antigen presentation properties of mature DCs are typically performed only 9 hours after the inflammatory stimulus (Young et al., 2007).

We monitored FcγR expression before and after maturation using the monoclonal antibody 24G2. This antibody has specificity for both FcγRII and FcγRIII. As in previous studies (Regnault et al., 1999; Sallusto et al., 1995), we found that the FcγRII/III was down-regulated slightly in BMDCs upon maturation (Fig. 1A). This effect was must more modest in splenic DCs (Fig. 1B). In contrast, DEC-205 was highly up-regulated during maturation by BMDCs (Fig. 1A), but was relatively unchanged upon maturation by splenic DCs (Fig. 1B).

Internalization of Immune Complexes and Anti-DEC-205 Antibody in Immature and Mature DCs

Our initial aim was to determine if receptor-mediated uptake continues in mature DCs after macropinocytosis has ceased. We therefore confirmed that the maturation conditions that we chose indeed led to the down-regulation of macropinocytosis, indicated by the lack of uptake of soluble OVA (Fig. 2). This is in agreement with more extensive studies (Garrett et al., 2000; Sallusto et al., 1995). We elected to use down-regulation of macropinocytosis as a marker of maturation for future experiments, both to ensure that maturation was complete and that uptake of receptor-targeted ligands was not occurring through fluid phase uptake.

We next sought to determine whether immune complexes (ICs) and anti-DEC-205 antibody were internalized by mature BMDCs. To quantitatively measure internalization we turned to flow cytometry. ICs were generated from HRP and rabbit-anti-HRP antibodies. These were bound to the surface of mature and immature DCs at 4° C. After 30 min of incubation at 37° C (or at 4° C as a control), immune complexes remaining on the cell surface were detected with an anti-rabbit antibody. At this time point while the total signal from ICs remained stable, both mature and immature DCs had barely detectable levels of surface ICs (Fig. 3A). This indicates that nearly 100% of the immune complexes had been internalized after 30 min.

We used a different assay to detect internalization of DEC-205. After binding anti-DEC-205 antibody to the surface of immature and mature cells, we incubated the cells at 37° C or 4° C. After 30 min we incubated the cells in an acetic acid buffer to strip off remaining surface antibodies. Only internalized antibody would be protected. A control wash in PBS was also used so that the total amount of antibody that had bound the cells could be measured. The assay showed that virtually all of the DEC-205 antibody was protected from the acid wash after the 37° C incubation (Fig. 3B). Thus, the internalization of DEC-205 was essentially complete by both mature and immature DCs. We concluded that unlike macropinocytosis, receptor-mediated endocytosis is not down-regulated by maturation.

Localization of Internalized Immune Complexes and anti-DEC-205 Antibody

Next, to confirm internalization, and to determine where captured antigen is trafficked, we used confocal microscopy. Because such assays rely on the analysis of individual cells, we felt that it was necessary to ensure that we were not analyzing a minor contaminating population of immature cells. Therefore, we used FACS to sort a highly purified population of cells that was CD86 high and could not take up soluble OVA. This technique allowed us to ensure that 1) these cells were mature by two independent criteria and 2) any uptake was indeed via a receptor-mediated pathway (Fig. 4).

After binding fluorescently-labeled ICs or anti-DEC-205 antibody to DCs at 4° C, the cells were warmed to 37° C and were fixed at various time points. We found that most ICs reached H-2M+ late endosomal/lysosomal compartments of mature DCs by 2 hours after internalization (Fig. 5A). In contrast, anti-DEC-205 antibodies partially localized to the lysosomal compartment after only 15 min, but exhibited a diffuse pattern after 2 hours, suggesting their degradation in or recycling from the lysosomal compartment (Fig. 5B).

This data is consistent with reports in other cell types. The FcγR has been shown to be degraded in the lysosomal compartment of macrophages after trafficking from the plasma membrane (Mellman et al., 1983). In contrast, DEC-205 recycling is in agreement with previous analysis of DEC-205 dynamics in transfected cells (Mahnke et al., 2000).

Next, we moved to immuno-electron microscopy to confirm the localization of internalized immune complexes and anti-DEC-205 antibodies. Fusion with the lysosomal compartment would be consistent with what has been observed in immature DCs(Chow and Mellman, 2005). We found that 60 min after internalization ~50% of immune complex-gold (Fig. 6) and anti-DEC-205-gold (Fig. 7) were detected in LAMP-2+ lysosomal compartments. Thus, mature DCs can efficiently internalize and deliver receptor-bound antigen to late endocytic compartments, where MHCII peptide complexes are known to form in immature or maturing DCs.

Phagocytosis of IgG Coated Latex Beads by Mature DCs

Phagocytosis is a physiologically important route of entry that permits DCs to present pathogen-derived antigens with high efficiency on MHCI and MHCII. It is also one that is down regulated during maturation due in part to a decrease in active, GTP-loaded Cdc42 (Cox et al., 1997; Garrett et al., 2000). Pathogens such as *S. typhimurium* can overcome this block by injecting a guanine nucleotide exchanger for Cdc42 (Garrett et al., 2000), suggesting that phagocytosis might also be triggered in mature DCs by coupling the phagocytic ligand to an appropriate signaling receptor. We hypothesized that the FcγR could be such a receptor as the C-terminal domain contains an ITAM that participates in FcR-mediated phagocytosis (Swanson, 2008). We therefore tested whether FcγR cross-linking could trigger phagocytosis in mature DCs.

As expected, both BSA and IgG-coated latex beads were avidly taken up by immature DCs and targeted to the lysosomes (Fig. 8A). BSA-coated beads were frequently found to be associated with mature DCs, but these were not internalized, as shown by confocal microscopy (Fig. 8A). This confirms previous findings that constitutive phagocytosis of untargeted particles is down-regulated by DC maturation (Garrett et al., 2000; Reis e Sousa et al., 1993; Wilson et al., 2006). In contrast, mature DCs internalized IgG-coated beads with comparable efficiency to immature DCs. As with immature DCs, IgG coated beads were trafficked by mature DCs to lysosomal compartments (Fig. 8A). We quantified this by

examining at least 90 randomly chosen cells from each condition, using lysosomal localization as an unequivocal marker of internalization (Fig. 8B).

These data indicate that while mature DCs down regulate non-specific forms of endocytosis (both macropinocytosis and phagocytosis), receptor-mediated endocytosis and phagocytosis remain efficient routes of uptake. Internalized cargo is trafficked to the lysosomal compartment where it would theoretically be available to antigen-processing machinery.

Discussion

We have demonstrated that in contrast to the prevailing view that mature DCs lose the ability to acquire new antigens, these cells can continue to efficiently capture and internalize antigens using the FcγR and DEC-205 receptors and traffic these antigens to the lysosomal compartment.

Mature BMDCs efficiently shut down macropinocytosis after maturation (Fig. 2). This fits conveniently with a model of mature DCs as antigen presenters, wary of taking up new antigens lest they present "self" as "foreign" and initiate autoimmunity (Reis e Sousa, 2006; Thery and Amigorena, 2001). MHCII, arguably the most important of the DC surface molecules, appears frozen on the surface of mature DCs. However, the plasma membrane of the mature DC is much more dynamic than this molecule would suggest. Unlike MHCII which has surface expression controlled by ubiquitination (Shin et al., 2006; van Niel et al., 2006), neither DEC-205 nor the FcγR have maturation-linked mechanisms regulating surface internalization.

The FcγR has a well-established physiologic role in both clearing immune complexes and presenting antigens (Takai, 2005). Interestingly, the receptor is slightly down-regulated upon maturation (Fig. 1). However, this did not prevent it from efficiently mediating the internalization of immune complexes and opsonized particles (Fig. 3, Fig 5, Fig. 7 & Fig. 8).

DEC-205 has a less established physiologic function, though it has been implicated as playing a role in the uptake of apoptotic cells (Shrimpton et al.,

2009). Its up-regulation during maturation (Fig. 1) makes it an intriguing choice for further study. It is not immediately apparent why it would be advantageous for a mature DC to have the increased ability to take up what may be primarily self-antigens.

Most studies of DC phagocytosis fail to make distinctions among the several mechanisms of phagocytosis, only some of which are well-characterized (Kerrigan and Brown, 2009). The constitutive phagocytosis that immature DCs display does not to require interaction with an endocytic receptor and is functionally similar to macropinocytosis. Immature DCs are capable of internalizing even large particles through this mechanism (Fig. 8). However, when macropinocytosis is down-regulated as a consequence of maturation, so is the non-specific internalization of large particles.

During FcγR-mediated phagocytosis, binding to IgG leads to signaling events initiated by the cytoplasmic domains of the FcγR which results in the receptor-guided advance of membrane and cytoskeleton over the surface of the particle (Swanson, 2008). Our data indicate that FcγR-mediated phagocytosis can occur long after the maturation stimulus (Fig. 8). While there is contradictory literature showing that even opsonized particles are not taken up by mature DCs, all of this work was done with cells matured by dissociation from lymphatic tissue (Steinman and Swanson, 1995). More work needs to be done to explore whether different modes of maturation lead to differential regulation of antigen uptake.

It seems that while immature cells have at least two distinct mechanisms of phagocytosis at their disposal, mature DCs have only receptor-mediated phagocytosis. Thus, DCs are selective in modulating cellular activities in response to maturation. They specifically down-regulate non-specific uptake, while leaving intact the internalization and trafficking of surface receptors. However, the consequences of such internalization in a cell type presumed functionally incapable of further antigen processing remains unclear.

Materials and Methods

Generation of BMDCs

C57BL/6J mice were purchased from The Jackson Laboratory. BMDCs were prepared by harvesting bone marrow from 6-12 weeks in age. Red blood cells were lysed in ACK lysis buffer (Invitrogen). Stem cells were isolated and incubated with anti-CD8 (TIB211), anti-CD4 (GK1.5), anti-CD45R (B220) and anti-MHCII (TIB120) before incubation at 37° C with rabbit complement (Rockland Immunochemicals). Cells were resuspended at 10e6 cells/ml in tissue culture medium. This medium contained RPMI 1640 (Invitrogen) supplemented with 5% FBS 2mM L-glutamine, 20μg/ml gentamicin, and supernatant from J558L cells transfected with murine GM-CSF cDNA. In some experiments 10 ng/ml recombinant GMCSF (Peprotech) and 10 ng/ml IL-4 (Peprotech) was used with equivalent results. Resuspended cells were plated at a concentration of 10e6 cells per well in 24 well plates (Corning). Cells were grown at 37° C in 7% CO2 and media was changed every 2 days. Immature DCs were used on day 6 of culture. Mature DCs were stimulated with 50 ng/ml of LPS 20 hours before harvest.

Generation of Splenic DCs.

Splenic and lymph node DCs were obtained from the spleens or lymph nodes of mice that had been injected IP with 3 μg of LPS (mature) or with PBS (immature). Tissue was cut into small pieces and incubated at 37° C with Liberase Blendzyme II

and DNase (Invitrogen) before cells were dissociated by passage through a 70 μm cell strainer (BD). When indicated, cells were purified with CD11c+ cell isolation beads after harvest (Miltenyi).

Cell Sorting

When indicated, cells were sorted before use. DCs were pulsed with OVA-Alexa-647, washed, and subsequently stained with anti-CD11c, and anti-CD86. Cells were washed and resuspended in PBS supplemented with 0.5% BSA at a concentration of 20e6 cells/ml. Cell were passed through a 70μm cell strainer sorted using a BD FACSVantage SE, BD FACSAria, or a Dako MoFlo.
Mature DCs were gated on CD11c+, CD86 high, soluble OVA-Alexa-647 low cells. Immature DCs were gated on CD11c+ CD86 low, soluble OVA intermediate to high cells.

Confocal Microscopy

BMDCs were seeded on alcian blue-coated coverslips at 37° C in serum free media for 15 min. Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cell were blocked and permeabilized in a buffer containing 10% goat serum, 10mM Hepes, pH 7.4, 10 mM glycine and 0.05% saponin. Cells were stained with a rabbit anti-H-2M polyclonal antibody and anti-MHCII antibody (K4H7) for 30 min. Cells were washed, then stained with anti-

mouse-Alexa633 or anti-rabbit-Alexa555. Imaging was performed with a Zeiss LSM 510 or Leica SP5 confocal microscope.

Electron Microscopy

Cells were labeled at 4° C with anti-DEC-205 biotin or anti-HRP-biotin/HRP immune complexes for 15 min. Cells were washed and labeled with 10 nm anti-biotin-gold (Aurion). Cells were fixed in suspension in 4% PFA, 0.25M HEPES buffer, pH 7.4 for 1 h at room temp. Concentration of PFA was increased to 8% in the same buffer and cells were stored overnight. Cells were then embedded in 10% gelatin, infiltrated in 2.3 M sucrose in PBS and frozen in liquid nitrogen. 70 nm cross-sections were cut on a Leica Ultracut cryo-microtome and collected on 100 hexagonal nickel grids coated with formvar and carbon. Sections were labeled with anti-MHC class II (TIB-120) or anti-LAMP-2 followed by rabbit anti-rat antibody and 5 nm protein A gold, then incubated with 1.8% methyl cellulose and 0.5% uranyl acetate and air dried. They were examined with a Tecnai 12 Biotwin electron microscope (FEI). Images were recorded using a Morada CCD camera (Olympus Soft Imaging Solutions). For quantitation approximately 30 images were taken at random. The percentage of DEC-205 gold or immune complex-gold contained within LAMP-2 or MHCII labeled structures were counted.

Flow Cytometry

Cells were resuspended in PBS containing 0.5% BSA, stained for 15 min on ice with antibodies conjugated with FITC, PE or APC, then washed twice with buffer. The stained cells were fixed with 1% PFA, then subjected to flow cytometry (BD Canto-II, FACSCalibur, or Aria). The collected data were analyzed with FlowJo software.

Endocytosis Assays

HRP/anti-HRP immune complexes were made by incubating HRP (Sigma) with anti-HRP-FITC or anti-HRP-Cy5 (Jackson ImmunoResearch Laboratories) at a 1:3 molar ratio on ice for 30 min. To detect antibody internalization using an acid wash assay, cells that had previously been bound with anti-DEC-205-Alexa 488 (Serotec) and incubated for 30 min at 37°C or 4°C were either incubated for 5 min in an acid buffer (0.2 M acetic acid, 0.15M NaCl, pH 3.0) to remove surface-bound antibodies or in PBS as control.

Phagocytosis Assays

1μm Fluoresbrite® yellow-green carboxylated latex microspheres (Polysciences Inc.) were coated overnight in 1mg/ml mouse IgG (Jackson ImmunoResearch Laboratories) or 1mg/ml BSA (Sigma) and washed 3 times in PBS before incubation with DCs at a concentration of 9.1×10^7 beads/ml.

Antibodies

Mouse IgG, anti-HRP-Cy5, anti-HRP-biotin were purchased from Jackson ImmunoResearch Laboratories. Anti-CD86-FITC/ PE/APC (GL1), anti-CD8a-PerCP (53-6.7), anti-CD45R-Pacific Blue (RA3-6B2), anti-CD11c-PE-Cy5 (HL3), anti-FcγII/III-PE (2.4G2), anti-MHCII (KH74) were purchased from BD. Anti-DEC-205-Alexa 488 were purchased from Serotec. Anti-DEC-205-PE was purchased from Miltenyi. Anti-H-2M (Pierre et al., 1997) is rabbit polyclonal antibody. Anti-MHCII (TIB-120) and anti-LAMP-2 were grown from hybridomas in the laboratory.

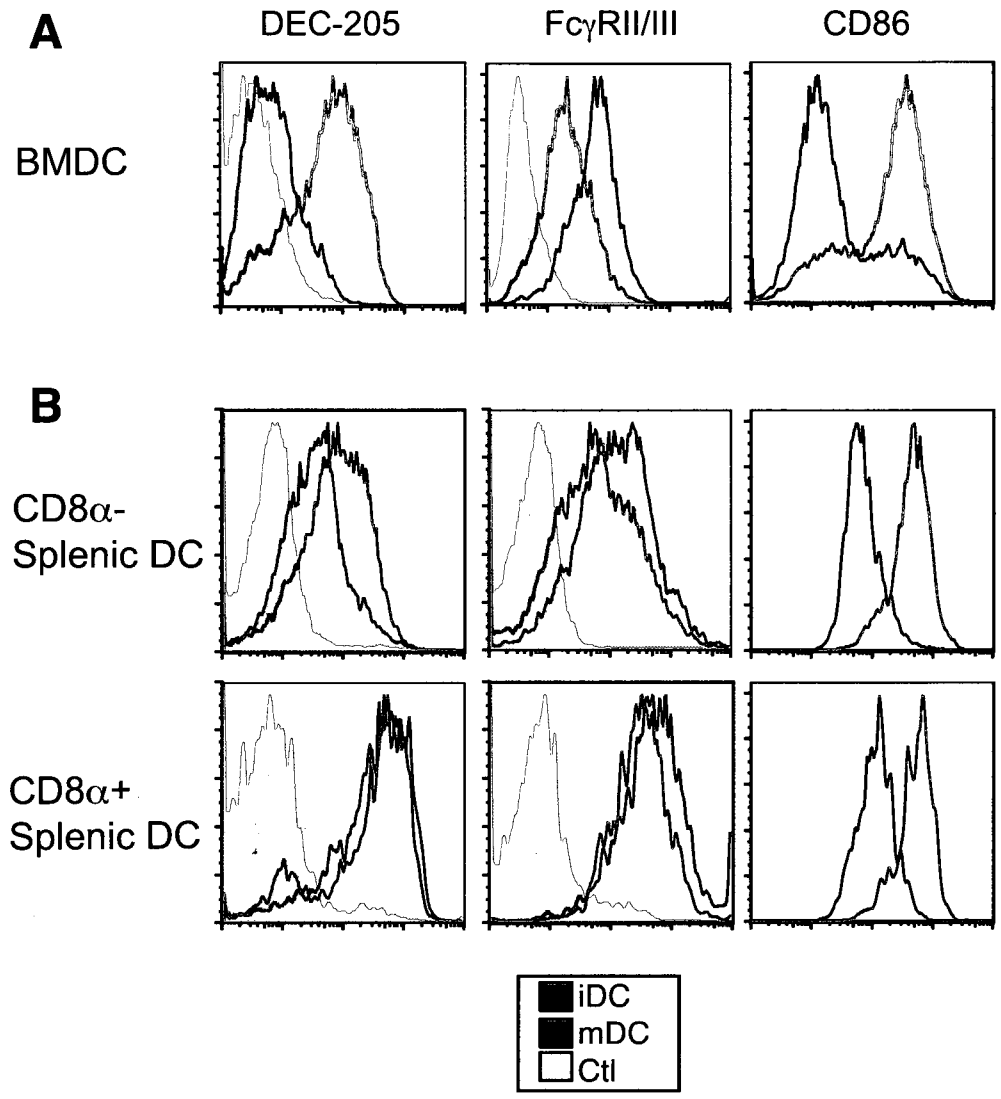

Figure 1 - Expression of DEC-205 and FcγRII/III by immature and mature DCs.
(A) BMDCs were treated with 50 ng/ml of LPS on the 5th day of culture for 20 h or left untreated. They were stained with anti-DEC-205, anti-FcγRII/III or isotype control as well as anti-CD86 and anti-CD11c. In DEC-205 and FcγRII/III plots, immature cells are gated on the CD11c+, CD86 low population while mature cells are gated on the CD11c+, CD86 high population. CD86 plots are gated on all CD11c+ cells
(B) Splenic DCs were derived from LPS treated (3 μg i.p.) or PBS treated control mice. 6 h after injection, spleens were harvested, and splenocytes isolated. Plots are gated on CD11c+ cells and CD8α+ or CD8α- populations as indicated.

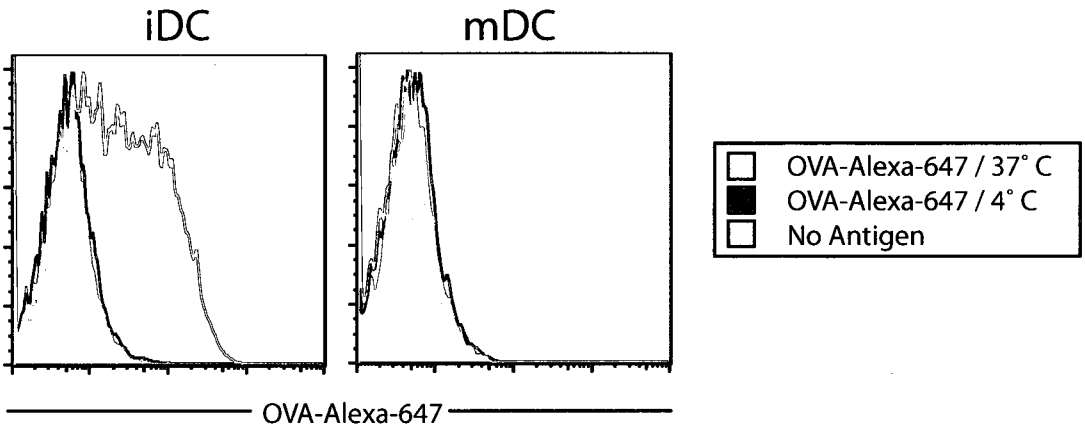

Figure 2 - Mature DCs down-regulate macropinocytosis.
BMDCs were treated with 50ng/ml of LPS on the 5th day of culture for 20 h or left untreated. Cells were incubated with OVA-Alexa-647 for 10 min at 37° C or on ice. Cells were washed, then stained for CD86 and CD11c and were analyzed by flow cytometry. Histograms of immature DCs are gated on CD11c+ CD86 low cells. Histograms of mature DCS are gated on CD11c+ CD86 high cells.

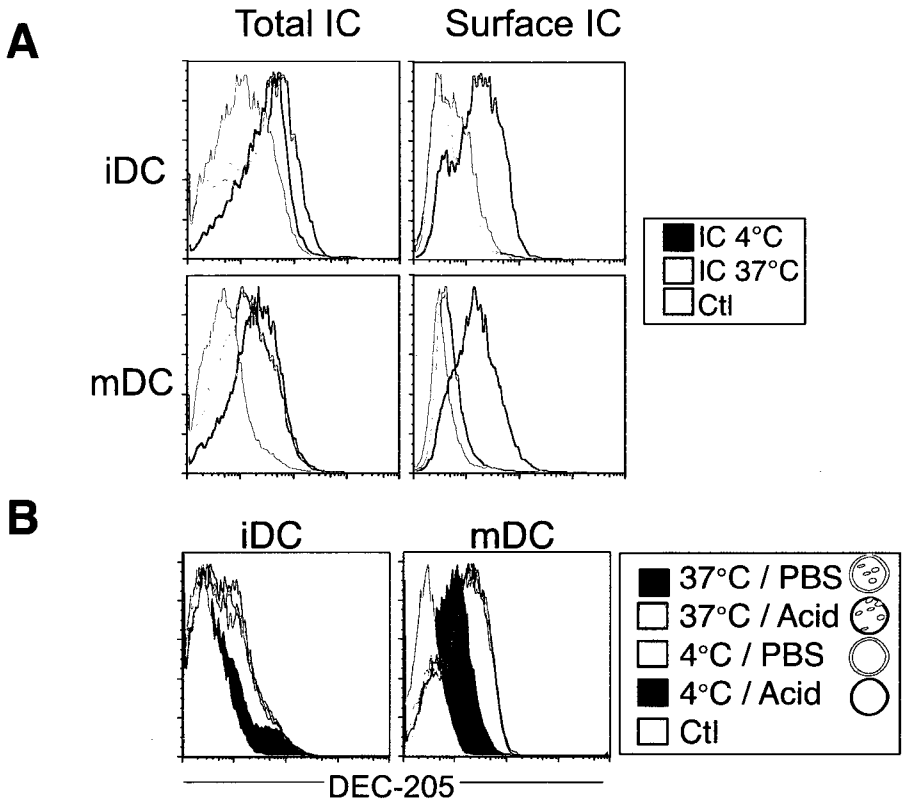

Figure 3 - Efficient internalization of immune complexes and anti-DEC-205 antibody by mature DCs.

(A) BMDCs were treated with 50 ng/ml of LPS for 20 h to induce maturation or left untreated. Cells were incubated with Cy5-labeled HRP/rabbit anti-HRP immune complexes (10μg/ml) for 15 min, then washed. Complexes remaining on the surface after 30 min of incubation at 4° C or 37° C were detected with Alexa488-labeled anti-rabbit IgG. Cells were labeled with anti-CD86 and anti-CD11c and were analyzed by flow cytometry. Histograms of immature cells are gated on CD86 low, CD11c+ cells. Histograms of mature cells are gated on CD86 high, CD11c+ cells.

(B) Immature or LPS-matured BMDCs were incubated with anti-DEC-205-Alexa488 (5 μg/ml) for 15 min. Cells were washed and incubated for 30 min at 4° C or at 37° C to allow for internalization. Antibodies remaining on the cell surface were removed by an acid buffer (internalized antibody is protected). Cells were labeled with anti-CD86 and anti-CD11c and were analyzed by flow cytometry. Histograms of immature cells are gated on CD86 low, CD11c+ cells. Histograms of mature cells are gated on CD86 high, CD11c+ cells.

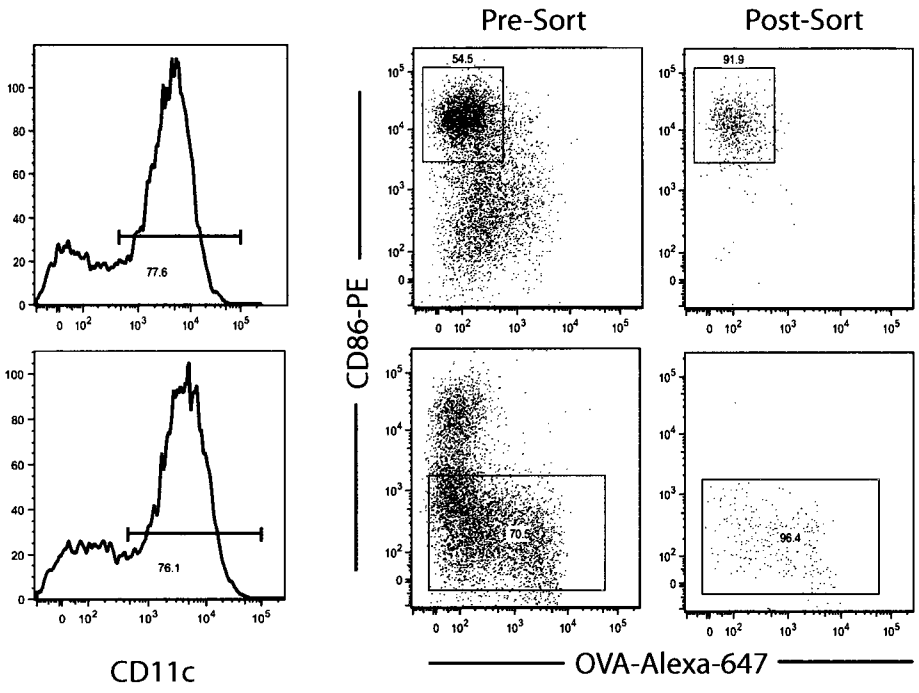

Figure 4 - Sorting pure populations of immature and mature DCs.
BMDCs were stimulated with 50 ng/ml of LPS for 20 h or left untreated. Immature and LPS-matured cells received a 10 min pulse with OVA-Alexa-647 (0.5 μg/ml) to measure macropinocytosis, and were stained with anti-CD11c and anti-CD86. Cells were then sorted by CD86 surface expression and level of OVA uptake. Mature DCs met the criteria of being CD11c+, CD86 high, and OVA-Alexa647 low. Immature DCs were CD11c+, CD86 low, and had internalized varied amounts of OVA.

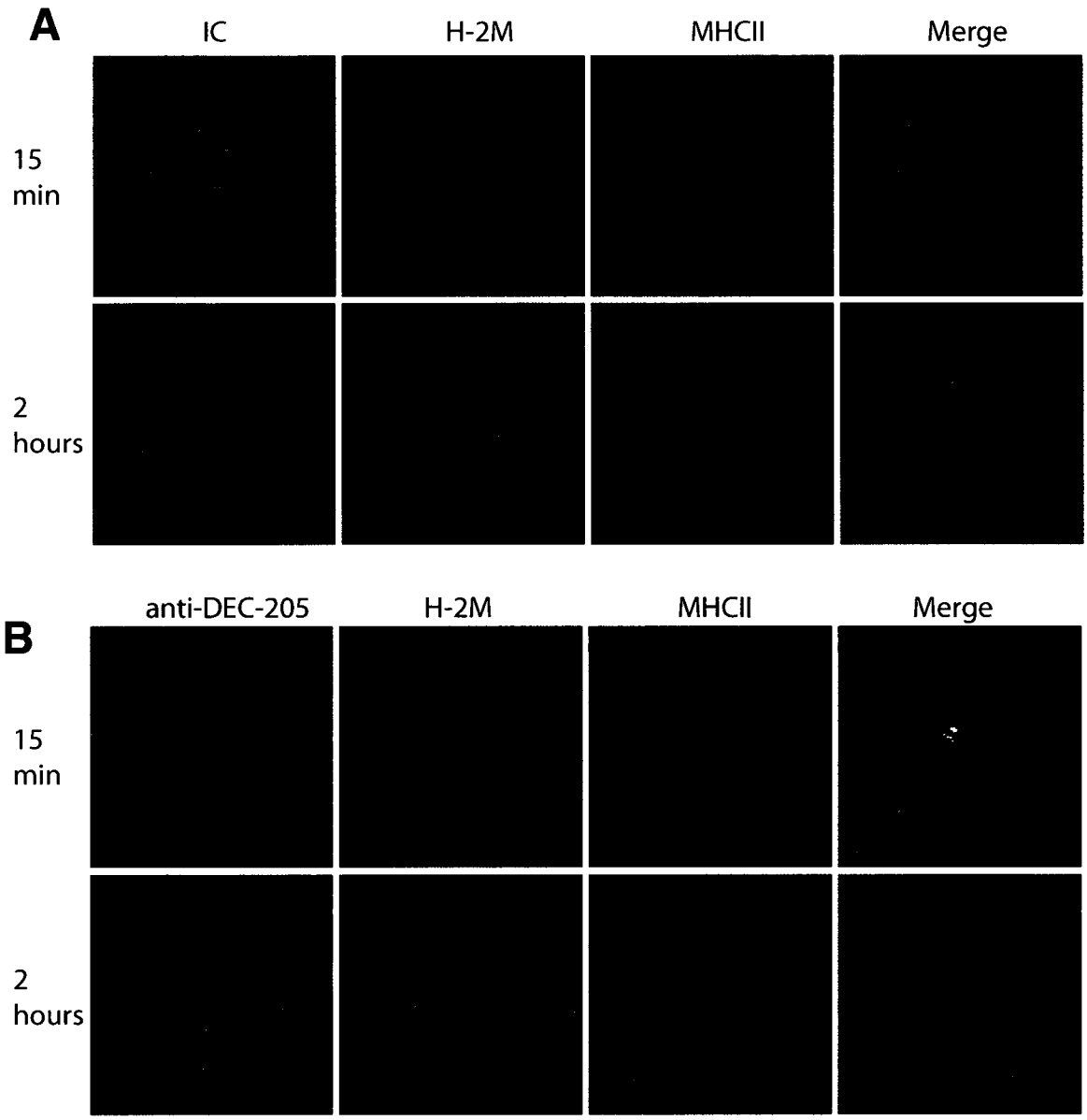

Figure 5- Efficient internalization of ICs and anti-DEC-205 antibody by mature DCs.

(A) LPS-matured BMDCs were sorted as in Fig. 4. They were then incubated with FITC-labeled immune complexes (5 μg/ml) at 4° C for 15 min. Cells were washed, and incubated at 37° C for the indicated time to allow for internalization before fixation. They were labeled with anti-H-2M and anti-MHCII and analyzed by confocal microscopy.

(B) LPS-matured BMDCs were sorted as in Fig. 4. They were then incubated with anti-DEC-205-Alexa488 (5μg/ml) at 4° C for 15 min. Cells were washed, and incubated at 37° C for the indicated time to allow for internalization before fixation. They were labeled with anti-H-2M and anti-MHCII and analyzed by confocal microscopy.

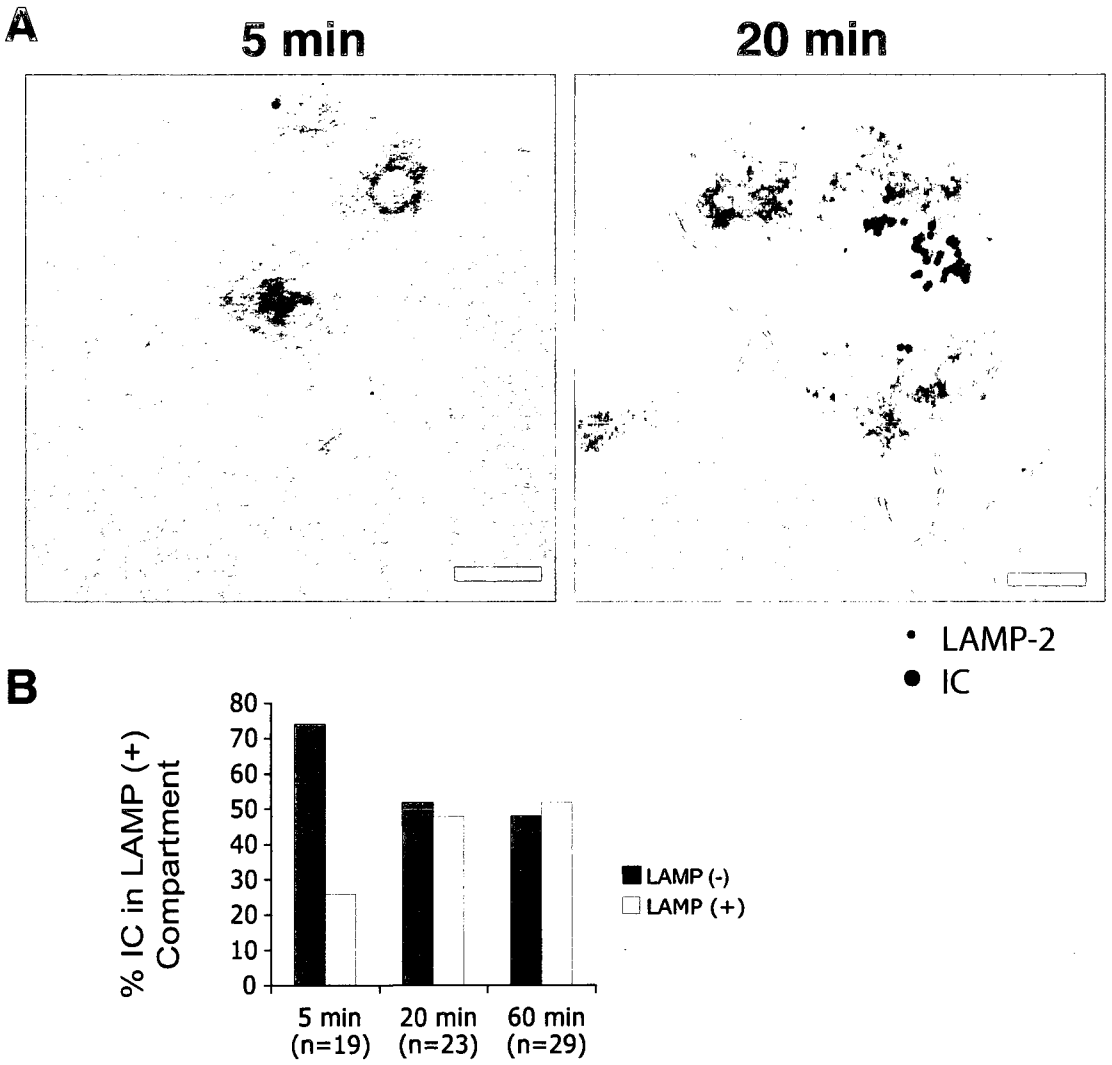

Figure 6- Trafficking of immune complexes to the lysosomal compartment.
(A) LPS-matured BMDCs were sorted according to the criteria described in Fig 4. Cells were incubated with HRP/anti-HRP-biotin immune complexes (10 μg/ml) at 4° C for 15 min, washed, then labeled with anti-biotin 10 nm gold at 4° C for 15 additional min. Cells were washed again, and incubated at 37° C for the indicated time to allow for internalization before fixation. Cryosections were labeled with anti-LAMP-2 and were examined by electron microscopy.
(**B**) 30 random images were acquired of fields containing at least one immune complex-gold containing vesicle. These vesicles were identified as LAMP-2 positive or negative compartments at 5 min, 20 min or 60 min after internalization.

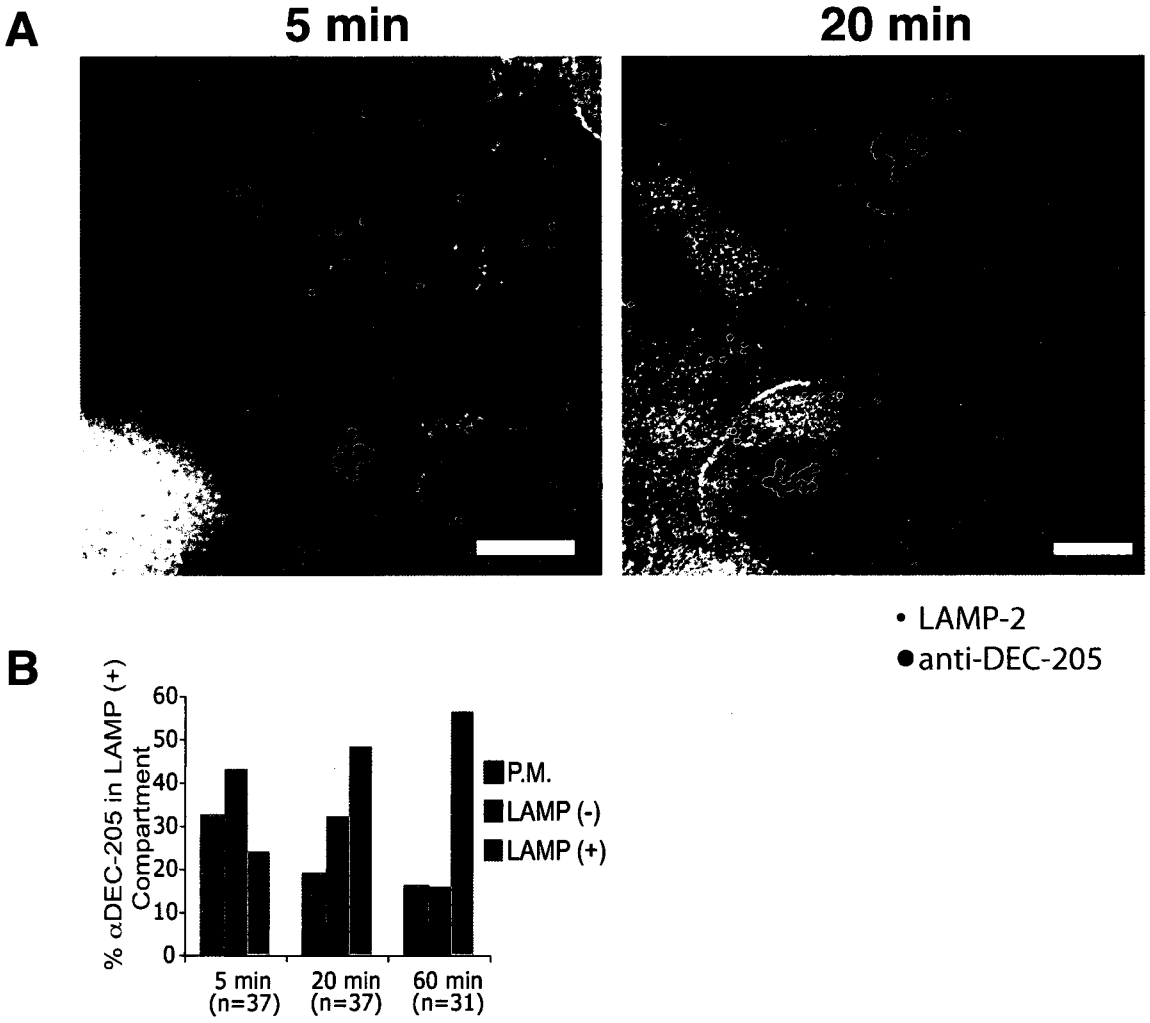

Figure 7 -Trafficking of anti-DEC-205 antibody to the lysosomal compartment.
(A) LPS-matured BMDCs were sorted according to the criteria described in Fig 4. Cells were incubated with anti-DEC-205-biotin (5 μg/ml) at 4° C for 15 min, washed, then labeled with anti-biotin 10 nm gold at 4° C for 15 additional min. Cells were washed again, and incubated at 37° C for the indicated time to allow for internalization before fixation. Cryosections were labeled with anti-LAMP-2 and were examined by electron microscopy.
(B) 30 random images were acquired of fields containing at least one DEC-205-gold containing vesicle. These vesicles were identified as LAMP-2 positive or negative compartments at 5 min, 20 min or 60 min after internalization.

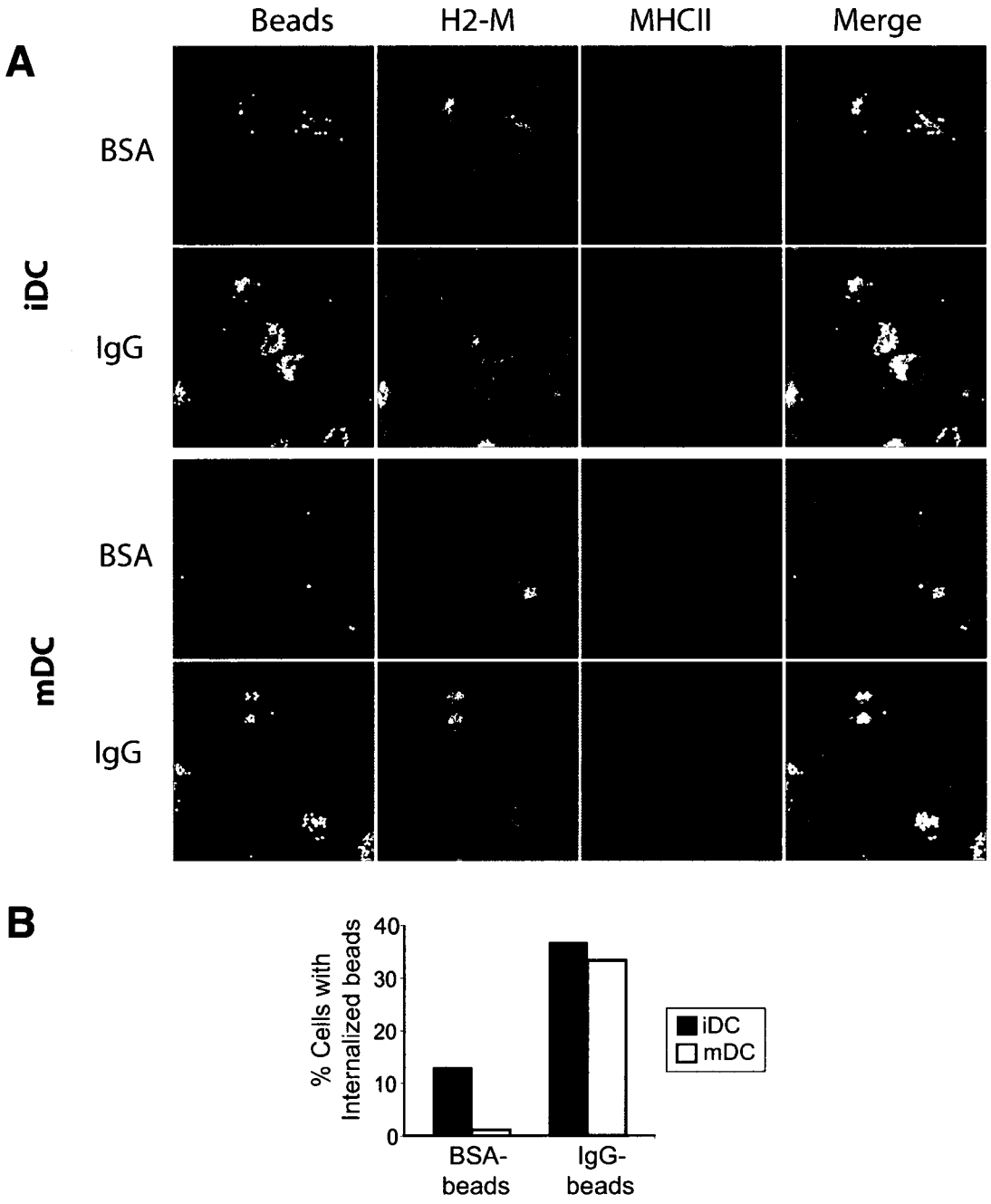

Figure 8 - Efficient phagocytosis of IgG-coated latex beads by mature DCs.
(A) Immature and LPS-matured BMDCs were incubated with IgG- or BSA-coated fluorescent beads, for 30 min at 37° C, washed, and chased for 15 min at 37° C. After pulse-chase, the cells were fixed, stained for the indicated markers, and analyzed by confocal microscopy.
(B) At least 90 DCs from each condition were selected at random and examined for the presence of beads within H2-M+ compartments, thus confirming complete internalization.

Chapter 3: Antigen Processing and Presentation By Mature DCs

Introduction

The concept that mature DCs lose the ability to acquire, process and present new antigens has become part of the dendritic cell dogma (Reis e Sousa, 2006). It was long known that mature DCs were powerful antigen presenters yet quite weak at presenting newly encountered antigens (Romani et al., 1989; Steinman, 1991). As the cell biology of DC maturation was unraveled, several mechanisms emerged to explain this. First, the down-regulation of endocytosis that accompanies maturation was shown to be an important initial barrier (Banchereau and Steinman, 1998; Wilson et al., 2006; Wilson and Villadangos, 2005). Next, MHCII synthesis is reduced in mature DCs (Cella et al., 1997; Pierre et al., 1997; Villadangos et al., 2001; Young et al., 2007). Furthermore, as DCs mature, MHCII molecules are translocated from lysosomes to the plasma membrane, which greatly decreases acceptor MHCII for peptides that might be generated following residual antigen uptake (Sallusto and Lanzavecchia, 1994; Turley et al., 2000).

A recent study by the Villadangos group attributed the lack of antigen presentation of mature DCs exclusively to reduced MHCII synthesis (Young et al., 2007). While the authors acknowledged that macropinocytosis is shut down in mature DCs, they argued that antigen can still be delivered by pinocytosis, and that the antigen presentation ability of immature and pre-matured DCs can be compared using soluble antigens (Young et al., 2007). The uptake of endocytic

tracer appeared similar in both mature and immature DCs under the conditions that they used (Young et al., 2007). Still, since no analysis of subcellular targeting of antigen was done, it was not clear that the reduced presentation could be attributed purely to a reduction in MHCII synthesis.

We demonstrated in chapter 2 that mature DCs can continue to take up antigens by receptor-mediated endocytosis, and even transport these antigens to traditional antigen-processing compartments. We therefore sought to target antigens to these receptors to test the ability of immature and pre-matured DCs to process and present newly internalized antigens.

To do this we employed two independent methods. First, we chose to compare the ability of immature and mature DCs to present different forms of antigens to transgenic T cells. We used a well-established system by which DCs are capable of internalizing, processing and presenting ovalbumin (OVA) on MHCI and MHCII to transgenic T cells (Robertson et al., 2000).

To target antigens to the FcγR, we created immune complexes (ICs) by incubating soluble OVA protein with polyclonal rabbit anti-OVA antibodies (Fig. 9B). Similar antigen-antibody complexes, composed of self or foreign antigens, are present under physiologic conditions *in vivo* (Takai, 2005). To target the OVA protein to DEC-205 we generated chimeric anti-DEC-205 antibodies that had the antigenic peptides recognized by OT.I and OT.II transgenic T cells engineered into the C-terminus of the antibody (Fig. 9C). An isotype control antibody containing the peptides was also generated (Fig. 9D). Identical antibodies containing the HEL

protein (Hawiger et al., 2001), larger fragments of the OVA protein (Dudziak et al., 2007) or gag-protein (Bozzacco et al., 2007) have shown that this antibody is a highly efficient means of inducing an immune response.

Soluble OVA is taken up by both macropinocytosis as well as the mannose receptor (Burgdorf et al., 2007). However, since we have seen that uptake was essentially eliminated by mature DCs (Fig. 2) (Garrett et al., 2000) and since mannose receptor is significantly down-regulated upon maturation, we used soluble OVA as a model of an antigen that would not be recognized by endocytic receptors on mature DCs (Fig 9A).

For these assays, it was important to ensure that we were not detecting the presence of contaminating immature DCs in our mature DCs populations. We therefore sorted BMDCs that were matured overnight with LPS (or left untreated) by two criteria: expression of the costimulatory molecule CD86 and macropinocytosis (Fig. 3). To select the mature cells, DCs that had been exposed to LPS for 20 hours were first pulsed with a small amount of fluorescently labeled soluble OVA. These cells were washed, then stained with anti-CD86 and CD11c. Only CD11c+ cells that had no detectable soluble OVA uptake and had fully up-regulated CD86 after LPS treatment, were considered mature. Immature cells had no exposure to LPS but were otherwise treated identically, but gated on the large population of cells that had taken up varied amounts of soluble OVA and were CD86 low-intermediate.

A second, independent assay was also devised using the Eα/YAe system. The monoclonoal YAe antibody acts much like a T cell receptor in that it recognizes a fragment of the Eα protein only when this fragment is bound to MHCII (Murphy et al., 1992). This antibody can then be used to assess antigen presentation at the single-cell level by flow cytometry. Unlike transgenic T cells, which are influenced by factors such as costimulatory molecules and cytokines, the YAe antibody measures only MHC-peptide complexes.

In order to test our hypothesis that DCs would be able to present receptor-targeted antigen but not untargeted antigen, we initially used two different antigen formulations. We made ICs with aggregates of his-tagged Eα and anti-his antibodies (Fig. 11C) or used Eα aggregates alone as an untargeted antigen that should be taken up primarily by macropinocytosis (Fig 11B). Unlike OVA, which is isolated from hen eggs, the Eα protein that we used was derived from transgenic E coli. This protein is therefore unglycosolated and should not be taken up by lectins.

Results

Mature DCs Present Receptor Targeted but Not Soluble Antigens to T cells

BMDCs were sorted into highly purified mature and immature populations by the criteria as described in Fig. 3. They were then pulsed with soluble OVA, OVA immune complexes or anti-DEC-205-OVA for 30 min before the cells were washed and cocultured with OT.II CD4+ T cells specific for MHCII/OVA complexes. As expected, soluble OVA was efficiently presented to T cells by immature DCs, but not by mature DCs, reflecting the down-regulation of macropinocytosis (Fig. 10 A&B). When exposed to OVA immune complexes and anti-DEC-205-OVA however, mature DCs were even more efficient at activating T cells than immature DCs (Fig. 10A&B).

There was poor presentation of DEC-205-targeted OVA by immature DCs (Fig. 10B). This was likely a reflection of the low levels of DEC-205 present on immature DCs (Fig. 1). This was not the case for FcγR-targeted ICs since these are taken up efficiently by both by mature and immature DCs (Fig. 3A).

To determine if DCs matured by other maturation stimuli also presented receptor-targeted antigen, we induced maturation with CpG DNA which binds TLR9, and poly I:C which binds TLR 3. In each case, these stimuli prevented presentation of untargeted OVA but did not prevent the presentation of FcγR-targeted ICs (Fig. 10C), showing that antigen presentation by mature DCs is possible regardless of the TLR ligand encountered.

The superior presentation ability of mature DCs is surprising. While it is possible that they actually showed increased efficiency at MHCII peptide generation when compared to the initially immature DCs, there are other possible explanations as well. Since these mature DCs received the initial maturation stimulus approximately 20 hours before the immature DCs, it was likely that they had greater levels of surface MHCII and CD86 for much of the coculture period. To remove differential co-stimulation as a possible factor, we coated assay plates with anti-CD28 antibodies. This ensured that all T cells would receive equal costimulation. Still, because the T cell assay measures only the ability of DCs to stimulate naïve T cells, we could not be certain about the relative antigen processing abilities of these cell types.

Direct Quantitation of MHCII-peptide Formation in Mature DCs

To make this comparison, we set out to directly quantify the formation of MHCII-peptide complexes by immature and mature DCs. We were able to do this by making use of the YAe monoclonal antibody, which recognizes a peptide derived from the Eα protein (52-68) when bound to MHCII (Murphy et al., 1992).

We compared the ability of immature and mature DCs to present aggregates of recombinant Eα protein, which should be taken up non-specifically (Fig. 11B) or Eα immune complexes, which should be taken up by FcγR (Fig 11C). For controls, immature and mature DCs that had not been exposed to Eα protein were used. All cells were treated with LPS at the time that antigen was provided.

Immature DCs formed MHCII-peptide complexes from untargeted Eα protein significantly better than the previously matured DCs, most likely reflecting their different capacities for macropinocytosis (Fig. 12 A&B). However, immature and mature DCs were able to generate specific MHCII-peptide from Eα-immune complexes with comparable efficiency (Fig. 12A&B).

Since we had previously established that both immature and mature DCs efficiently internalize opsonized particles (Fig. 8), we used opsonized *E. coli* that express the Eα protein to compare the presentation of MHC-peptide complexes from this physiologically relevant source of antigen (Fig. 11A). Otherwise identical bacteria that did not express the protein were used as a control. As was seen for presentation of ICs, both immature and mature DCs generated similar levels of MHCII-peptide complexes after phagocytosis of bacteria-associated antigen (Fig. 13). Thus, although mature DCs lose the ability to present untargeted antigens, their ability to present antigens on MHCII molecules following receptor-mediated uptake by clathrin coated pits or phagocytosis remains entirely intact.

Mature DCs Cross-present Receptor Targeted Antigens

Previous studies have suggested that mature DCs lose the ability to cross-present antigen on MHCI molecules, while retaining their ability to present endogenous antigens. This has been attributed to a lack of (non-specific) antigen internalization (Wilson et al., 2006), or poor translocation of antigen to the cytosol (Gil-Torregrosa et al., 2004). To assess cross-presentation, we returned to the OVA

system and targeted OVA to the FcγR and DEC-205 using OVA ICs or anti-DEC-205-OVA, respectively. We found that mature DCs cross-presented this antigen far more efficiently than immature DCs (Fig. 14).

While the mechanism of cross-presentation on MHCI is still poorly understood, the increased levels of MHCI synthesis in mature DCs (Delamarre et al., 2003) likely explains the level of cross-presentation if antigen is efficiently internalized. Our studies differ from previous work by making use of receptor-mediated rather than non-specific endocytosis (Wilson et al., 2006), or by using a more sensitive T cell assay that more clearly revealed the cross-presentation that was indeed observed earlier (Gil-Torregrosa et al., 2004).

Discussion

The model of the immature DC as antigen gatherer and the mature DC as antigen presenter is well established for a series of reasons. First, it makes sense in an immunological context, in that it can be invoked as a mechanism to prevent autoimmunity. Second, it is suggested by DC cell biology since maturation leads to both down-regulation of macropinocytosis and translocation of available MHCII from the loading compartment to the plasma membrane. And most importantly, there is *in vitro* and *in vivo* data showing that mature DCs no longer efficiently newly encountered antigens (Romani et al., 1989; Young et al., 2007).

Despite these past findings, relatively little work has been done to rigorously test this hypothesis. In agreement with previous literature, we found that DCs that had previously been matured with a variety of maturation stimuli did not efficiently present soluble OVA or Eα aggregates (Fig. 10 & Fig. 12). However, we found that if these antigens were targeted to DEC-205 (Fig. 10A) or the FcγR (Fig. 10B & Fig. 12) they were taken up and presented efficiently.

We measured presentation both with naïve T cells capable of recognizing OVA fragments in the context of MHCI or MHCII (Fig. 10 and Fig. 14) and with the YAe antibody, specific for MHCII-Eα peptide complexes (Fig. 12 and Fig. 13). The OVA system is well established, highly sensitive and physiologic in that the readout reflects T cell activation. In contrast, the Eα system only measures MHC-peptide complexes. While this is a less physiologic system, it is not influenced by differences such as costimulatory molecule surface expression or cytokine

production. Together, these data conclusively demonstrate that antigen processing and presentation is active in mature DCs.

These findings contradict the concept that mature DCs are specialized purely for antigen presentation of previously acquired antigens and play no further role in antigen acquisition. However, our data are not in direct conflict with any particular report. Past reports have looked at DCs matured by culture of dissociated lymphatic tissue rather than inflammatory stimulus and/or have used antigens that should theoretically be taken up more efficiently by immature DCs (Romani et al., 1989; Young et al., 2007). We are not aware of any other studies that have targeted antigen to endocytic receptors to ensure adequate uptake.

Importantly, we confirmed that antigen was delivered to the same antigen-loading compartment in both immature and mature cells (Chapter 2). There have been reports of differential trafficking of antigen receptors upon maturation (Engering et al., 2002b) and of the route of antigen uptake influencing presentation (Burgdorf et al., 2007). For these reasons, it is vital to confirm that uptake and trafficking of antigen is equivalent in any study that attempts to compare the antigen processing ability of two cell types.

The finding alters the concept of the "snapshot hypothesis," the notion that a DC presents a "snapshot" of the antigenic environment at the time that maturation is induced (Villadangos et al., 2005). Perhaps this is taking the analogy too far, but a DC may act more like a camcorder, presenting a selection of antigens encountered after TLR stimulation. However, in this case antigen presentation

correlates with receptor expression. This gives us strong motivation to better characterize the roles of endocytic receptors and their modulation by inflammatory stimuli. The immunological implications of mature DCs presenting self or foreign antigens long after their initial activation are discussed later in this work.

Materials and Methods

Generation of BMDCs

As described in Chapter 2

Antibodies

Anti-CD86-FITC/ PE/APC (GL1), anti-CD8a-PerCP (53-6.7), anti-CD45R-Pacific Blue (RA3-6B2), anti-CD11c-PE-Cy5 (HL3), were purchased from BD. Anti-6xHis was purchased from Sigma and Serotec. Polyclonal rabbit anti-*E. coli*-FITC was purchased from Serotec. Anti-MHCII/Eα peptide (52-68)-biotin (YAe) was purchased from eBiosciences. Anti-OVA (Delamarre et al., 2003) is a rabbit polyclonal antibody. Anti-DEC-205-OVA and the isotype control-OVA (gifts from Ralph Steinman, Rockefeller University, NYC, NY) are hybrid antibodies containing the amino acid residues OVA 257-264 and OVA 323-329 at the C-terminus of the antibodies. They were purified from stably transfected lines of mouse myeloma cells using the LONZA recombinant protein expression system.

In Vitro T Cell Activation Assays

DCs were sorted as described above, and incubated for 30 min with DEC-205-OVA, OVA immune complexes or soluble OVA in the presence of 50 μg/ml of LPS (Sigma). Cells were washed and the indicated number of DCs were cocultured

$5x10^6$ CD4+ or CD8+ T cells that had been purified using negative selection (Miltenyi) from the spleens of OT.II or OT.I mice respectively. 24 hours later supernatants were collected and IL-2 concentration was measured by ELISA to determine the degree of T cell activation.

Alternatively, DCs were incubated with CFSE labeled T cells for 60 h. T cells were stained with anti-CD4 antibody and were analyzed by flow cytometry to determine the extent of cell division. CD4+ T cells were gated and CFSE dilution was assessed. The percentage of dividing cells was determined for each sample. Anti-DEC-205-OVA was a gift from Ralph Steinman and was prepared from stably transfected lines of mouse myeloma cells using the LONZA recombinant protein expression system. Immune complexes were made from polyclonal rabbit anti-OVA antibodies mixed at a 3:1 molar ratio with OVA (Worthington). Soluble OVA (Worthington) was purified by centrifugal filtration (Millipore) or size-exclusion chromatography (Bio-Rad) to remove impurities and pre-formed peptides.

Quantitation of IL-2 Production by ELISA

Maxisorb 96 well plates (Nunc) were coated with anti-IL-2 mAb in a phosphate buffer, ph 9.0 overnight. Washes were performed with 0.01% tween in PBS. Plates were blocked in 2% BSA for and washed before supernatants from T cell activation and IL-2 standards (Peprotech) were applied to the plates. Bound IL-2 was detected with anti-IL-2-biotin antibodies (BD) followed by streptavadin-HRP

and addition of a TMB substrate (Pierce). Absorbance at 405 nm was determined using a microplate reader (Molecular Devices).

Generation of Recombinant I-Eα Protein

E. coli expressing 6xHis-tagged I-Eα protein was a kind gift of Ruslan Medzhitov. The bacteria was grown in 2 L of LB in the presence of 100 μg/ml ampicillin and 30μg/ml kanamycin. When the OD reached 0.6, IPTG was added to a final concentration of 1mM. Bacteria were grown for 6 more hours, centrifuged and resuspended in 200ml of 8M urea pH 8 containing protease inhibitor cocktail (Roche) and 20mM imidazole. After stirring at room temperature for 4 hours the lysate was spun and the supernatant was incubated with nickel agarose beads (Qiagen). The beads were washed with 8M urea (pH 6.3) and loaded into a chromatography column (Bio-Rad). The recombinant protein was eluted with 8M urea (pH 4.5). The eluate was dialyzed against PBS using a 10,000 MWCO Slide-A-Lyzer Cassette (Pierce). Insoluble aggregates were separated from soluble protein by centrifugation. Eα–immune complexes were created by mixing Eα aggregates with anti-his antibody on ice (Sigma or Serotec) at a 1:3 molar ratio.

Flow Cytometry

Cells were resuspended in PBS containing 0.5% BSA, stained for 15 min on ice with antibodies conjugated with FITC, PE, PE-Cy7, pacific blue, PE-Cy5 or APC, then washed twice with buffer. The stained cells were fixed with 1% PFA, then

subjected to FACS (BD Canto-II, FACSCalibur, or Aria). The collected data was analyzed with FlowJo software.

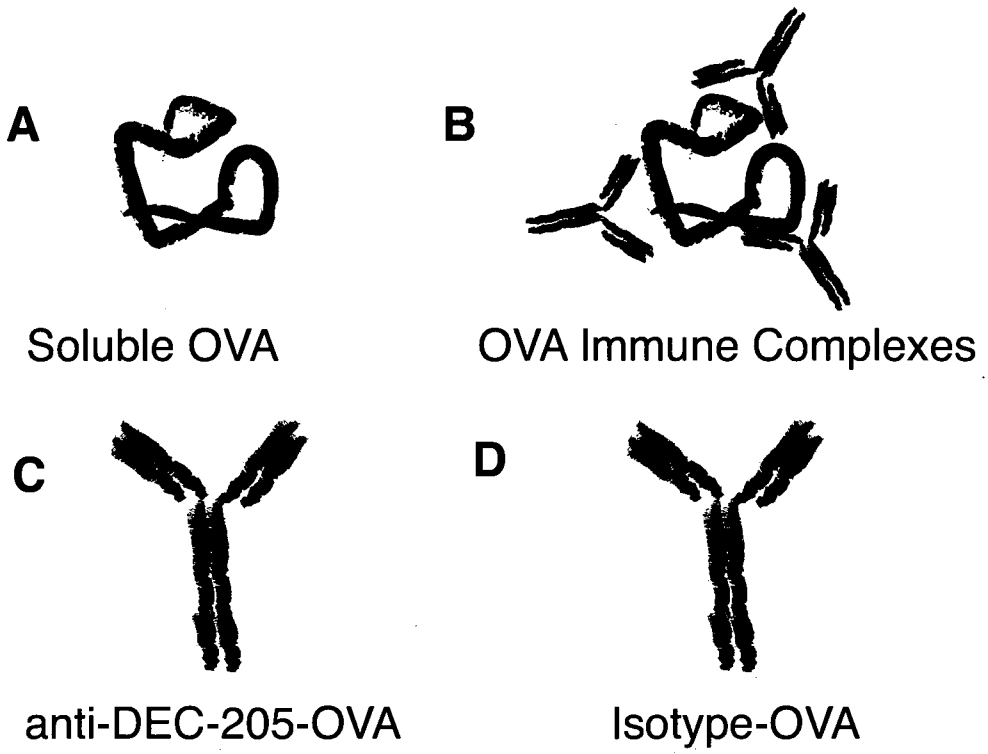

Figure 9 - Antigens used to assess T cell activation by immature and mature DCs.
(A) OVA was purified from contaminants and pre-formed peptides by centrifugal filtration or size-exclusion chromatography
(B) OVA immune complexes were generated by incubating OVA with rabbit-anti-OVA polyclonal antibodies at a 1:3 molar ratio.
(C) Anti-DEC-205-OVA and **(D)** isotype control antibodies are fusion proteins containing amino acid residues OVA 257-264 and OVA 323-329 at the C-terminus of the antibodies.

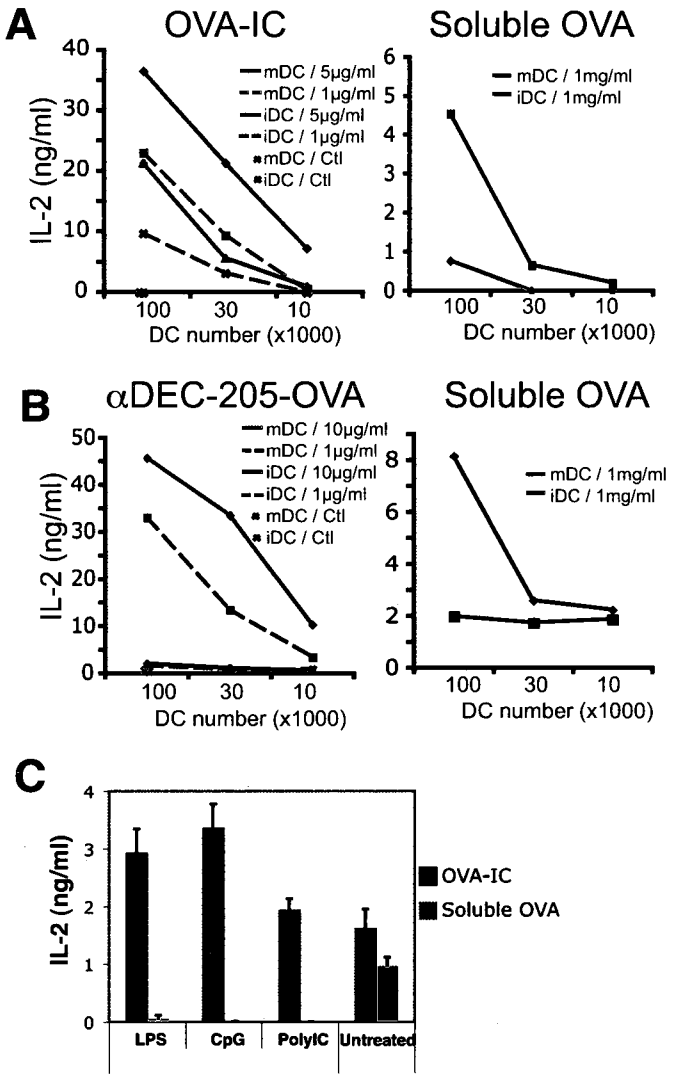

Figure 10 - Mature DCs efficiently present receptor-targeted antigens to CD4+ T cells.

(A) Immature and mature BMDCs were sorted as in Fig. 4. They were then incubated with OVA/rabbit anti-OVA immune complexes or soluble OVA for 30 min in the presence of LPS at 37° C. DCs were washed and cultured with 10e5 CD4+ OT.II T cells for 24 h. T cell activation was assessed by measuring IL-2 secretion by ELISA. Averages of triplicates are displayed.

(B) Experiments performed as in (A) but with anti-DEC-205-OVA and isotype control-OVA fusion proteins.

(C) BMDCs were cultured for 20 h in the presence of 50 ng/ml LPS, 10 µg/ml poly I:C, 10 nM CpG DNA or left untreated. The cells were incubated with OVA/rabbit anti-OVA immune complexes or soluble OVA for 30 min. DCs were washed and cultured with 10e5 CD4+ OT.II T cells for 24 h. IL-2 secretion was measured by ELISA. Experiment performed in triplicate. Averages of triplicates are displayed. Error bars indicate standard deviation.

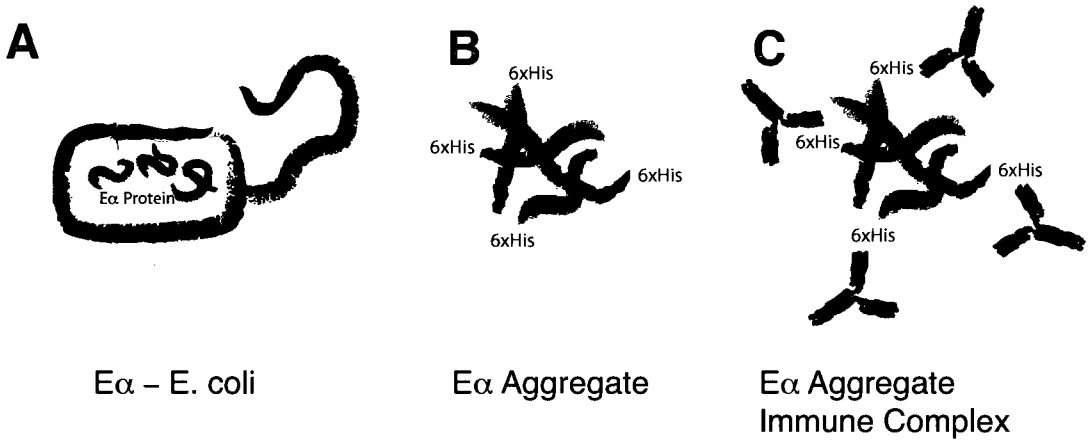

Figure 11 - Model antigens used to quantitiatively measure MHCII-peptide complex generation.
(A) Eα synthesis was induced in transgenic *E. coli* using IPTG.
(B) Aggregates of Eα were purified from the transgenic *E. coli*.
(C) Immune complexes of Eα were made by incubating Eα aggregates with anti-6xHis monoclonal antibodies.

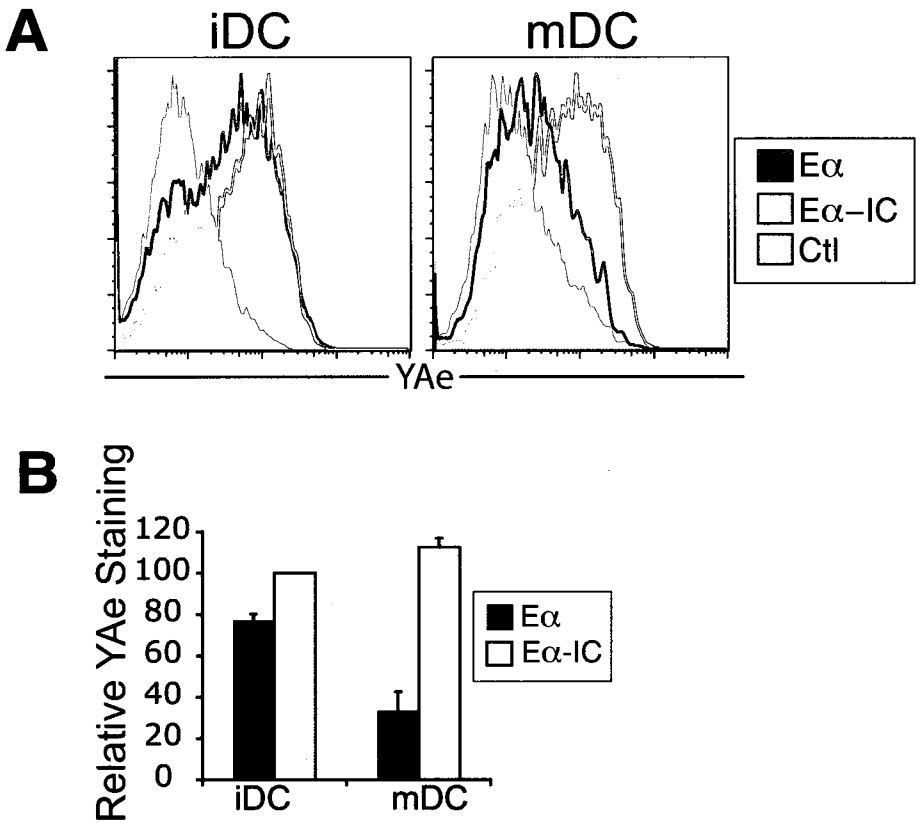

Figure 12 - Mature DCs efficiently generate MHCII-peptide complexes.
(A) Immature or LPS-matured BMDCs were labeled with anti-CD86, then incubated with 30 μg/ml of Eα aggregates or 30 μg/ml Eα aggregate immune complexes for 5 h. Cells were then stained with YAe antibody and anti-CD11c and analyzed by flow cytometry. LPS-matured cells were gated on the CD11c+ CD86 high population. Immature cells were gated on the CD11c+ CD86 low population.
(B) MFI of YAe staining from 2 independent experiments. Bars indicate standard error.

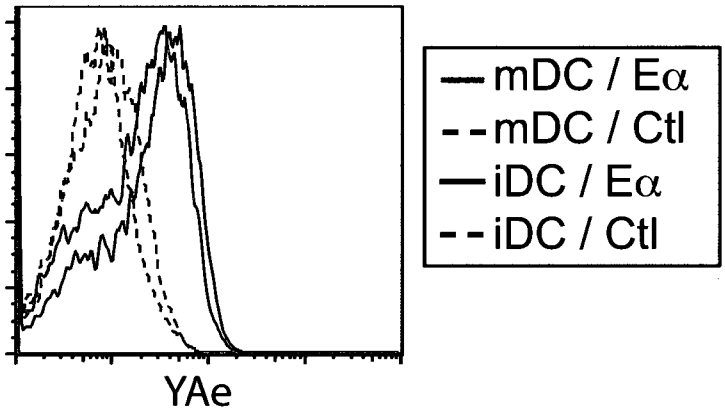

Figure 13 - Mature DCs efficiently phagocytose and present bacterial antigens.
Immature or LPS-matured BMDCs were labeled with anti-CD86, then incubated at a 1:100 ratio with opsonized E. coli that had been induced with IPTG to express Eα. Uninduced bacteria were used as a control. 7 h later, cells were stained with YAe antibody and anti-CD11c and analyzed by flow cytometry. LPS-matured cells were gated on the CD11c+ CD86 high population. Immature cells were gated on the CD11c+ CD86 low population.

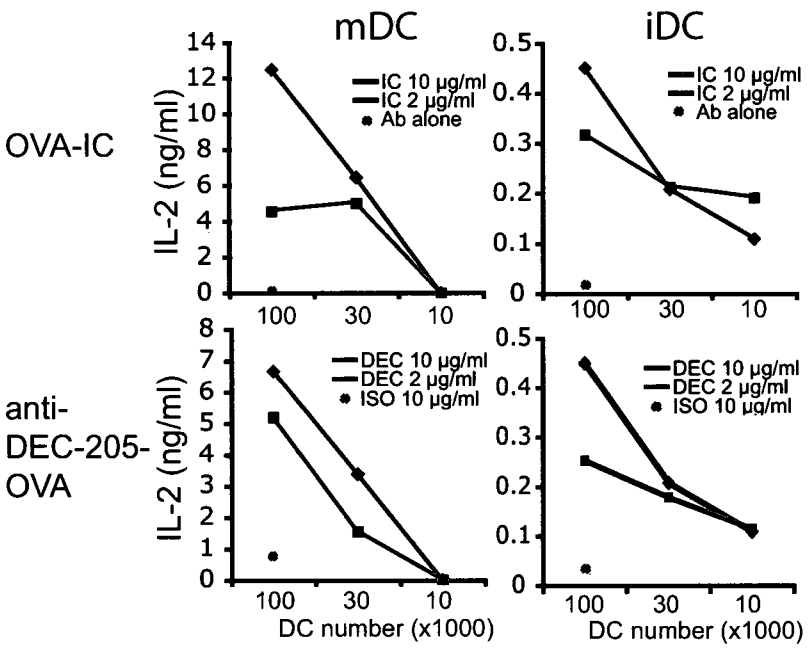

Figure 14 - Mature DCs efficiently cross-present receptor-targeted antigens to CD8+ T cells.
Immature and mature BMDCs were sorted as in Fig. 4. They were then incubated with OVA/rabbit anti-OVA immune complexes or soluble OVA for 30 min in the presence of LPS at 37° C. DCs were washed and cultured with 10e5 CD8+ OT.I T cells for 24 h. T cell activation was assessed by measuring IL-2 secretion by ELISA. Averages of triplicates are displayed.

Chapter 4: Mechanism of Antigen Presentation By Mature DCs

Introduction

The maturation program has been described as designed to optimize antigen presentation while shutting down mechanisms related to acquisition and processing (Reis e Sousa, 2006). Our data indicates that while mature DCs are more selective in their antigen acquisition, they are quite efficient at antigen processing (Chapter 3). We therefore set out to determine by what mechanism mature DCs present antigens.

There are few known changes that occur during maturation that would be expected to interfere with efficient cross-presentation on MHCI. In fact, DCs are known to efficiently present new endogenous antigens long after the maturation stimulus was received (Wilson et al., 2006) so it seems that MHCI pathway is active in mature DCs. In addition, MHCI synthesis is up-regulated in mature DCs (Delamarre et al., 2003). It is therefore not entirely surprising that mature DCs would have the capacity to cross-present antigen on MHCI once provided with a receptor-dependent route of entry into endocytic compartments, andfrom there, translocation to the cytosol. For this reason, and because the mechanism of cross-presentation in immature DCs in unclear, we did not attempt to determine the mechanism of MHCI presentation in mature DCs in this study.

However, the mechanism of MHCII presentation is not immediately apparent. First, the location of antigen loading needs to be determined. While

immature DCs load antigen in the lysosomal compartment, the dramatic reorganization of MHCII to the cell surface upon maturation suggests that little if any MHCII remains available for loading in mature DCs (Boes et al., 2002; Chow et al., 2002; Kleijmeer et al., 2001; Pierre and Mellman, 1998; Pierre et al., 1997). We therefore set out to document whether internal stores of antigen could be detected upon closer inspection, and if so, where these stores are located.

Secondly, the role that the down-regulation of MHCII synthesis plays needs to be determined. This down-regulation has been observed previously (Cella et al., 1997; Pierre et al., 1997; Villadangos et al., 2001; Young et al., 2007) and is known to correlate with reductions in antigen presentation (Young et al., 2007). Still the reduction in MHCII synthesis has never been definitively shown to *cause* the reduction in antigen presentation. We therefore had two additional tasks. The first was to confirm that MHCII synthesis is down-regulated under the conditions that we have been using. The second was to determine the source of loaded MHCII in mature DCs.

Results

Visualization of Internal Stores of MHCII

The most notable difference between immature and mature DCs when inspected by immunofluorescence is the nearly complete transfer of MHCII from the lysosomal compartment to the plasma membrane (Fig. 5). Since immature DCs load MHCII in the lysosomal compartment (Chow and Mellman, 2005), we asked if there were residual internal stores of MHCII that could be visualized with a more sensitive method of detection. We hypothesized that high concentrations of MHCII on the plasma membrane might artifactually mask a reduced amount of MHCII in late endosomes or lysosomes.

We therefore set out to markedly reduce the surface signal so any residual internal stores might be revealed. We blocked surface MHCII with unlabeled antibody, then fixed and permeabilized DCs before detecting internal MHCII with labeled anti-MHCII antibody. Indeed, under these conditions, an internal pool of MHCII in mature DCs could be detected that colocalized with the lysosomal marker H-2M (Fig. 15).

Next we used electron microscopy to confirm that ICs internalized via the FcγR accumulated in MHCII-containing lysosomes over time. HRP-anti-HRP ICs were bound to the surface of sorted, mature DCs at 4° C, then warmed to 37° C and fixed at various time points after internalization began. Labeling for MHCII was performed. We found that by 60 min after internalization that approximately 80% of the ICs were localized to MHCII positive compartments (Fig. 16A&B). This data

indicates that both MHCII and newly internalized antigen are together in the same place at the right time for peptide loading to occur.

Monitoring of Internal MHCII-Peptide Formation

To more definitively determine whether peptide-loading can occur in the MHCII-diminished lysosomal compartment of mature DCs, we once again took advantage of the specificity of the YAe antibody for MHCII-Eα peptide complexes. We incubated mature and immature DCs with Eα immune complexes, which we had previously shown to be internalized and presented by both cell types (Fig.12). After 90 min, we washed the cells and either mounted and fixed them, or incubated them at 37° C for another 18.5 hours before fixation. We then used YAe antibody to track the formation and the distribution of the newly formed MHCII-peptide complexes over time.

By 90 min, both mature and immature DCs had the majority of MHCII-peptide complexes localized to the lysosomal compartment (Fig. 17). These complexes were translocated to the plasma membrane with further incubation (Fig. 17), as found previously for immature DCs that are induced to mature (Turley et al., 2000). We conclude that like immature DCs, mature DCs load antigenic peptides on MHCII in the lysosomal compartment and then make the MHCII-peptide complexes available on the plasma membrane for T cell recognition.

The Role of Newly Synthesized MHCII in Mature DCs

While mature DCs appear to continue to form MHCII-peptide complexes in the lysosomal compartment, the source of the MHCII is still unclear. Previous work has suggested that antigenic peptides can be loaded both on newly synthesized or on recycling MHCII molecules (Trombetta and Mellman, 2005). However the down-regulation of MHCII synthesis after maturation has been proposed as the mechanism responsible for reduced presentation of newly acquired antigens by mature DCs (Young et al., 2007). We therefore decided to document whether this down-regulation of MHCII was indeed occurring in our system, and if so whether it was complete.

We first examined the rate of MHCII synthesis in immature and mature DCs by metabolic labeling and autoradiography. As in the antigen presentation experiments, these cells were sorted to high purity to ensure that MHC production by mature DCs could be attributed to synthesis by this population rather than to contaminating immature DCs. Like previous studies, (Cella et al., 1997; Pierre et al., 1997; Villadangos et al., 2001; Young et al., 2007) we found significant but incomplete down-regulation of MHCII synthesis after maturation. MHCII synthesis was decreased by ~90% in mature BMDCs and 65% in splenic DCs (Fig. 18).

To determine the contribution of the residual de novo synthesis of MHCII to peptide loading, we used cycloheximide (CHX) to arrest protein synthesis. We found that at doses as low as 1μM, there was nearly complete inhibition of protein synthesis, yet that this was fully reversible after washout, showing that the cells

were not killed by this dose of CHX (Fig 19A). Immature and mature DCs were pulsed with CHX 30 for min before the cells were pulsed with Eα immune complexes. The CHX pulse prevented DCs from using newly synthesized MHCII to load peptides. 5 hours later, MHCII peptide surface presentation was detected with YAe.

We found that CHX treatment significantly impaired, but did not completely abrogate, the formation of YAe-reactive MHCII-peptide complexes in immature DCs, demonstrating the contribution of both newly synthesized and recycled MHCII to peptide loading (Fig. 19B). Interestingly, CHX had little effect in fully mature DCs, indicating that recycled or previously synthesized MHCII plays a major role in the presentation of newly captured antigens (Fig. 19B).

Discussion

Here we show that the mechanism of antigen presentation by mature DCs has both similarities and differences to the mechanism in immature DCs. As with immature DCs, antigen is trafficked to the lysosomal compartment (Fig. 16). This is where we visualized the formation of MHCII-peptide complexes in both cell types (Fig. 19). However, we confirmed a significant reduction in MHCII synthesis by mature DCs (Fig. 18) (Cella et al., 1997; Pierre et al., 1997; Villadangos et al., 2001; Young et al., 2007). This reduction was not complete, so there was theoretically still newly synthesized MHCII available for loading (Fig. 18). However, administration of CHX led to a much greater reduction in presentation by immature DCs, suggesting that newly synthesized MHCII plays a smaller role in presentation by mature DCs (Fig. 18).

This could indicate that recycling MHCII plays a larger role in antigen presentation by mature DCs. However, recycling is problematic for several reasons. First, although the recycling pathway is well-established with certain antigens, there is evidence that most antigens are efficiently presented on newly synthesized rather than recycled MHCII (Geuze, 1998; Pinet et al., 1995). Testing the ability of mature DCs to present antigens other than OVA and Eα would be helpful but would still leave another issue unresolved: the marked stabilization of MHCII on the plasma membrane of mature DCs by a loss of ubiquitination (Shin et al., 2006). Maturation leads to the stabilization of MHCII on the cell surface, with

the MHCII half-life increasing at least 10-fold (Cella et al., 1997). This long half-life makes surface MHCII a less likely reservoir for newly acquired antigens.

Still, while ubiquitin signals the sorting of MHCII molecules to late endosomes and lysosomes (Shin et al., 2006), it is less clear that it is necessary for the initial step of endocytosis. This is true not only for MHCII but also for receptors such as the EGF receptor for which the role of ubiquitin is quite well characterized (Sorkin and Goh, 2009; Stahl and Barbieri, 2002). Thus, even in mature DCs, a small level of MHCII endocytosis might be expected to continue. The size of the intracellular pool would undoubtedly remain small under steady state conditions.

The small intracellular pool of MHCII that we observed (Fig. 15) appeared sufficient for effective antigen processing and presentation (Chapter 3). However it is minimal compared to the large intracellular pools of MHCII observed in immature DCs (Fig. 17, top row)(Turley et al., 2000). Is it possible that such large pools of MHCII are not essential for efficient antigen presentation? Studies of B cells and B cell lines may suggest that this is the case as they too are efficient antigen presenters with only small intracellular pools of MHCII (Trombetta and Mellman, 2005).

Perhaps we will gain more insight into the function of mature DCs from B cells. Both cell types must recognize of antigen via endocytic receptors for efficient uptake. Both have high levels of surface MHCII while having relatively small stores of internal MHCII.

Materials and Methods

Generation of BMDCs

As described in Chapter 2

Antibodies

Mouse IgG, anti-HRP-Cy5, anti-HRP-biotin were purchased from Jackson ImmunoResearch Laboratories. Anti-CD86-FITC/ PE/APC (GL1), anti-CD8a-PerCP (53-6.7), anti-CD45R-Pacific Blue (RA3-6B2), anti-CD11c-PE-Cy5 (HL3), anti-MHCII-biotin/unlabeled (KH74), MHCII (TIB-120) and streptavidin-PE were purchased from BD. Anti-6xHis was purchased from Sigma and Serotec. Anti MHCII/Eα peptide (52-68)-biotin (YAe) was purchased from eBiosciences. Anti-H-2M (Pierre et al., 1997), anti-MHCI (Delamarre et al., 2003) were rabbit polyclonal antibodies. Secondary antibodies were purchased from Molecular Probes.

Metabolic Labeling

BMDCs or splenic DCs ($5x10^6$) were pre-incubated in RPMI without met/cys (Invitrogen) for 15 min, then proteins were labeled with medium containing S^{35}-met/cys (Amersham) for 20 min. Cells were lysed in buffer containing 1% Triton X-100, then subjected to immuno-precipitation of MHCII and MHCI proteins using TIB-120 and a rabbit polyclonal respectively. The proteins were loaded to SDS-PAGE and imaged by phospho-image analyzer (Amarsham-Pharmacia Typhoon).

Confocal Microscopy

BMDCs were seeded on alcian blue-coated coverslips at 37° C for 15 min. Cells were washed with PBS and fixed with 4% paraformaldehyde. Cell were blocked and permeabilized in a buffer containing 10% goat serum, 10mM Hepes, pH 7.4, 10 mM glycine and 0.05% saponin. Cells were stained with a rabbit anti-H-2M polyclonal antibody, anti-MHCII antibody (K4H7) and YAe-biotin. Secondary antibodies were obtained from Molecular Probes. Imaging was performed with a Zeiss LSM 510 or Leica SP5 confocal microscope.

Electron Microscopy

Cells were labeled at 4°C with anti-HRP-biotin/HRP immune complexes for 15 min. Cells were washed and labeled with 10 nm anti-biotin-gold (Aurion). Cells were fixed in suspension in 4% PFA, 0.25M HEPES buffer, pH 7.4 for 1 hr at room temp. Concentration of PFA was increased to 8% in the same buffer and cells were stored overnight. Cells were then embedded in 10% gelatin, infiltrated in 2.3 M sucrose in PBS and frozen in liquid nitrogen. 70 nm cross-sections were cut on a Leica Ultracut cryo-microtome and collected on 100 hexagonal nickel grids coated with formvar and carbon. Sections were labeled with anti-MHC class II (TIB-120) or anti-LAMP-2 followed by rabbit anti rat antibody and 5 nm protein A gold, then incubated with 1.8% methyl cellulose and 0.5% uranyl acetate and air dried. They were examined with a Tecnai 12 Biotwin electron microscope (FEI). Images were recorded using a Morada CCD camera (Olympus Soft Imaging Solutions). For

quantitation approximately 30 images were taken at random. The percentage of DEC-205 gold or immune complex-gold contained within MHCII labeled structures were counted.

Flow Cytometry

Cells were resuspended in PBS containing 0.5% BSA, stained for 15 min on ice with antibodies conjugated with FITC, PE, PE-Cy7, pacific blue, PE-Cy5 or APC, then washed twice with buffer. The stained cells were fixed with 1% PFA, then subjected to FACS (BD Canto-II, FACSCalibur, or Aria). The collected data was analyzed with FlowJo software.

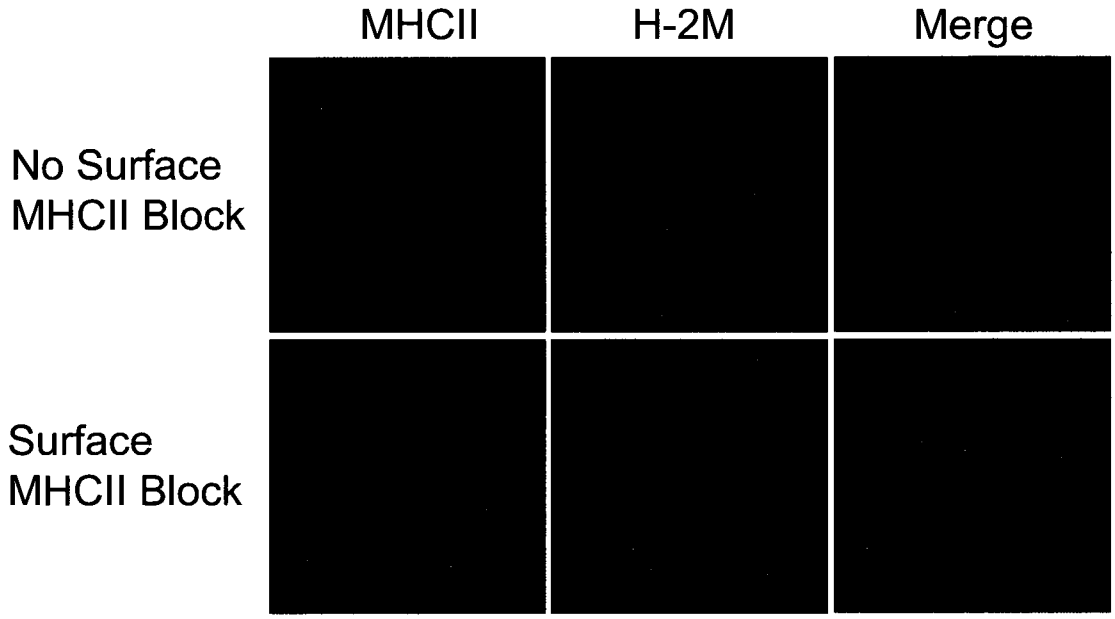

Figure 15 - Detection of intracellular MHCII positive compartment.
LPS-matured BMDCs were blocked with 10 μg/ml of unlabeled anti-MHCII (bottom) or not blocked (top) before fixation in 4% PFA. Cells were then permeabilized, stained with anti-MHCII and anti-H2-M and analyzed by confocal microscopy.

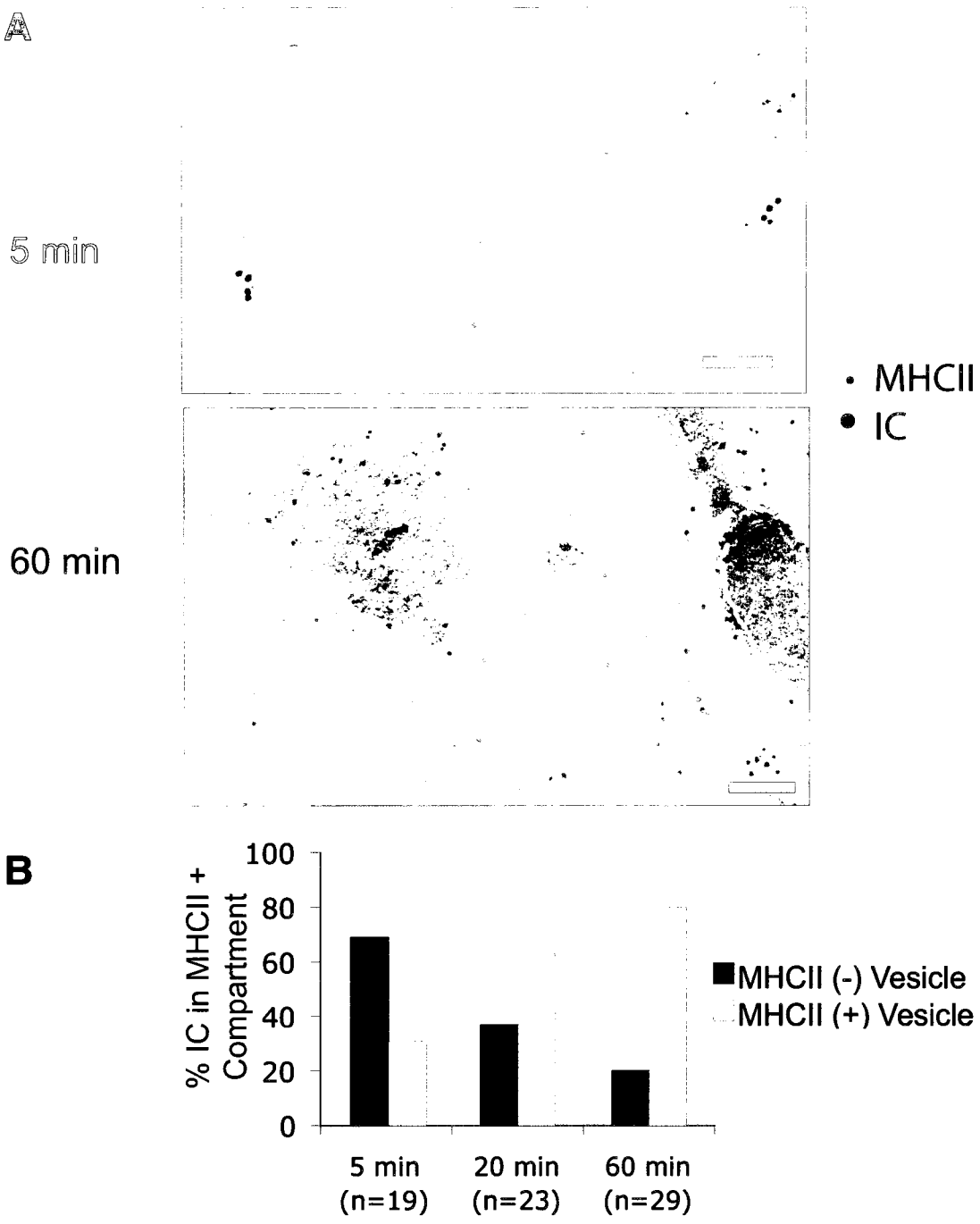

Figure 16 - Mature DCs traffic antigen to lysosomes containing MHCII.
(A) LPS-matured BMDCs were sorted as described in Fig. 4 and labeled with HRP-anti-HRP immune complexes-biotin for 15 min, washed, then labeled with anti-biotin gold for 15 min. Cells were incubated at 37° C for the indicated times before fixation. Scale bars, 100 nm.
(B) 30 random images were acquired of fields containing at least one immune complex-gold containing vesicle. These vesicles were identified as MHCII positive or negative compartments at 5 min, 20 min or 60 min after internalization.

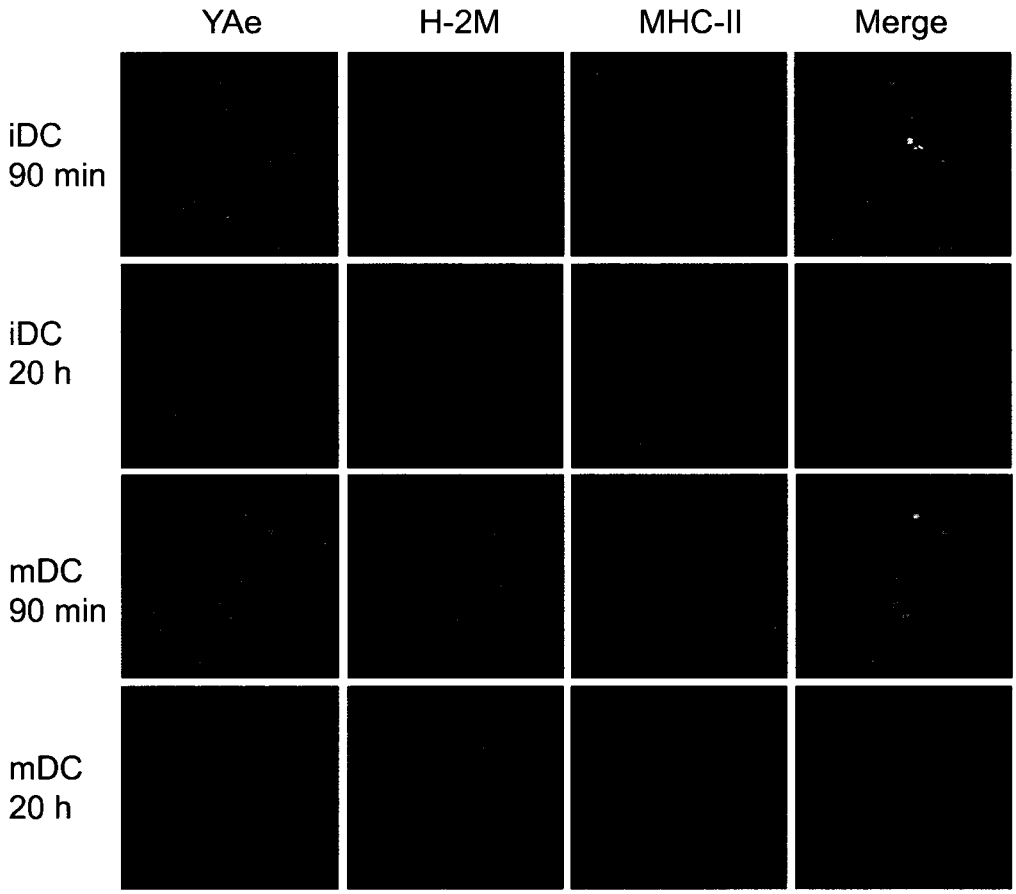

Figure 17 - Mature DCs generate MHCII-peptide complexes in the lysosomal compartment.
Immature or LPS-matured BMDCs were sorted as in Fig. 4 and incubated at 37° C for 90 min with Eα ICs (30 μg/ml) and LPS. Cells were washed, then fixed or incubated at 37° C for an additional 18.5 h before fixation. Cells were stained with YAe, anti-H-2M and anti-MHCII antibodies, and analyzed by confocal microscopy.

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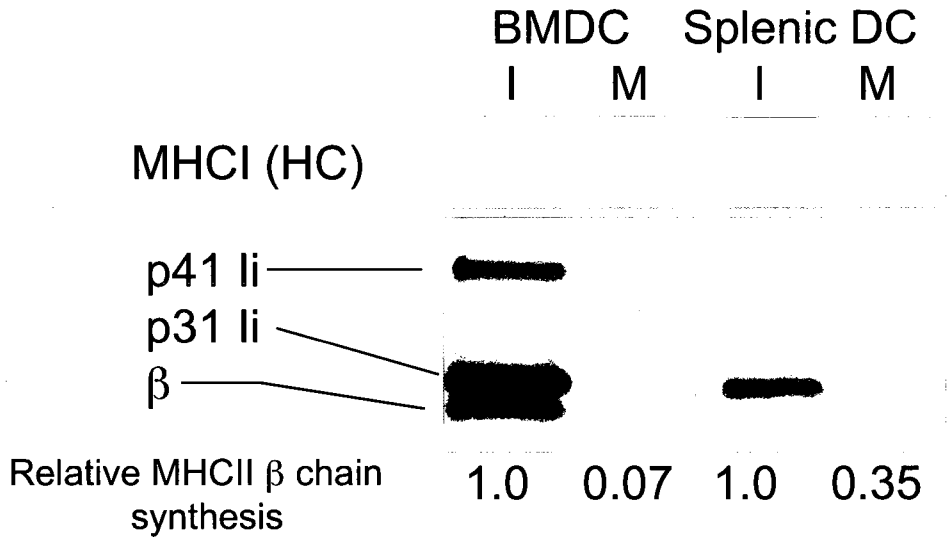

Figure 18 - Mature DCs partially down-regulate MHCII synthesis. Immature or LPS-matured BMDCs or splenic DCs from LPS or PBS injected mice were sorted as in Fig. 4. They were then incubated for 15 min in cys/met free media before addition of met/cys S35 for 20 min. Cells were washed in cold PBS supplemented with 1% FBS and lysed in triton lysis buffer. MHCII and MHCI were immunoprecipitated sequentially from lysates and run in a 10% SDS/PAGE and visualized by autoradiography.

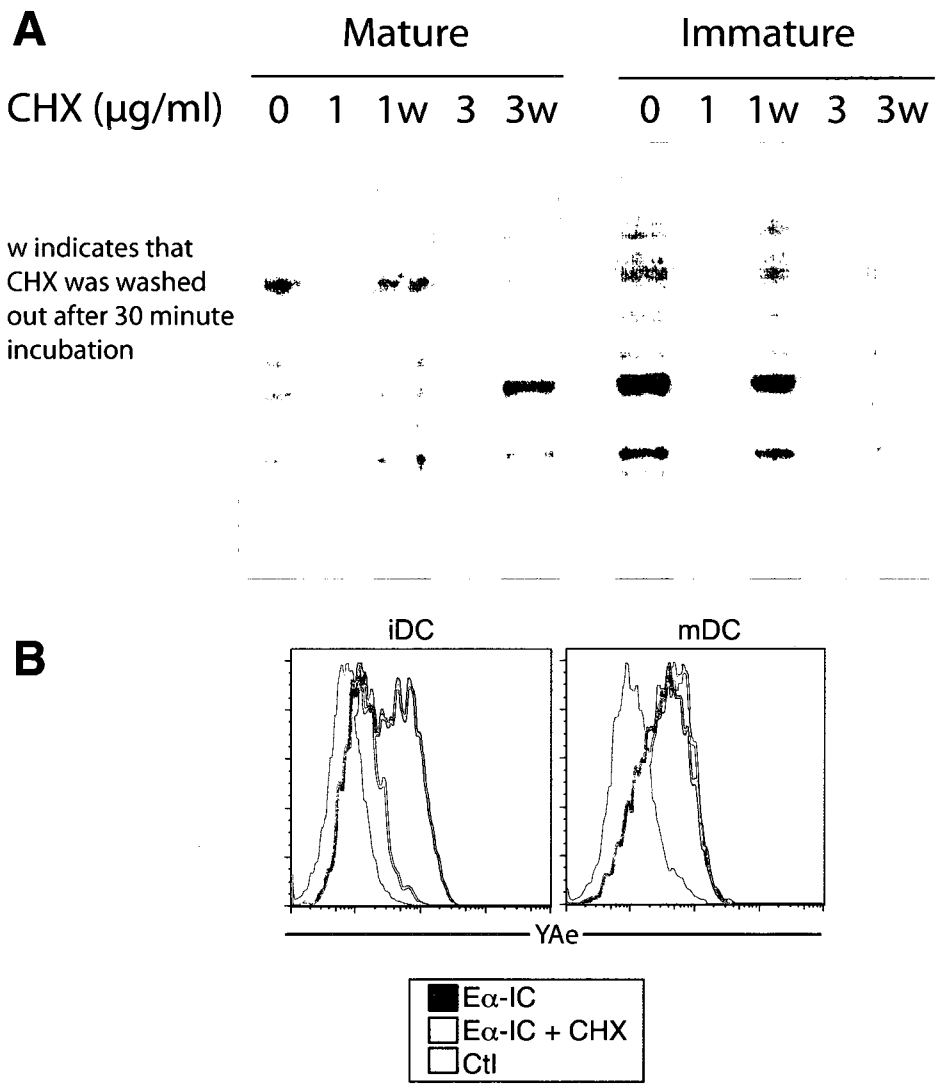

Figure 19 - Inhibition of protein synthesis significantly inhibits MHCII-peptide generation in immature but not mature DCs.
(A) Immature or LPS-matured BMDCs were pretreated with the indicated amount of CHX for 30 min. In indicated samples, CHX was washed out before all samples were incubated for 15 min in cys/met free media (with or without CHX) before addition of met/cys 35S for 20 min. Cells were washed in cold PBS supplemented with 1% FBS and lysed in triton lysis buffer. Lysates were run on a 10% SDS/PAGE and visualized with a phospho-imager.
(B) Immature or LPS-matured BMDCs were stained with anti-CD86, then incubated with CHX (1 µg/ml) for 30 min. Eα immune complexes (30 µg/ml) were added to the DCs for 5 h before staining with YAe antibody and anti-CD11c. Cells were analyzed by flow cytometry. LPS-matured cells were gated on the CD11c+ CD86 high population. Immature cells were gated on the CD11c+ CD86 low population.

Chapter 5: *In Vivo* Antigen Capture and Presentation By Mature DCs

Introduction

The previous chapters have outlined how mature DCs present receptor-targeted antigens *in vitro* (Chapter 3) and suggested how this can occur mechanistically (Chapters 2 & 4). Next we decided to determine if these findings are applicable to the initiation of an immune response under inflammatory conditions *in vivo*.

There are several studies that have assessed the ability of mature DCs to present antigens *in vivo*. One study, examining the ability of pre-matured DCs to present antigens on MHCI, showed that while presentation of endogenous antigen persists, cross-presentation does not occur (Wilson et al., 2006). The authors attributed this to poor antigen uptake by mature DCs, as the lack of antigen presentation correlated with significantly decreased phagocytosis in these cells. Since we have seen that efficiently internalized antigens are cross-presented efficiently (Fig. 14), reduced antigen uptake is indeed the most likely explanation for poor cross-presentation *in vivo* under inflammatory conditions.

This same group has also assessed presentation on MHCII *in vivo*. They found that mature DCs did not activate transgenic T cells if the mice were primed with inflammatory stimuli before immunization with soluble OVA. In this case,

they attributed the lack of presentation not to poor antigen uptake, but to reduced MHCII synthesis (Young et al., 2007).

However, another possible explanation for their findings, and one that would be consistent with their previous study of MHCI presentation, is that the mature DCs did not efficiently internalize the OVA protein that was used as their model antigen. We therefore set out to devise an *in vivo* assay where we could measure presentation of receptor-targeted antigens by mature DCs after induction of systemic inflammation with LPS.

The Villadangos group used a common method of assessing antigen presentation *in vivo* involving the adoptive transfer of CFSE-labeled T cells (Gudmundsdottir et al., 1999; Wells et al., 1997; Young et al., 2007). Over the course of several days, activated T cells divide, each division leading to a dilution of the labeling dye. Such dilution can be assessed by flow cytometry at a single cell level, and can be accurately used to monitor the immune response to a given antigen.

Their group saw reduced T cell proliferation when antigen was administered after 9 hours of systemic inflammation, which they attributed to the reduced MHCII synthesis that accompanies DC maturation (Young et al., 2007). We decided to repeat this experiment with receptor-targeted antigens, yet felt however, that it would be difficult to interpret such an experiment using the same methodology. While the absence of proliferation arguably shows the lack of effective antigen presentation, the presence of proliferation could be due to either presentation by

mature DCs or presentation from a re-emerging population of immature DCs. While maturation is a terminal differentiation program, *in vivo* there are DC precursors that are theoretically ready to step in and play the role of a phenotypically immature DC. Over several days, it would be impossible to rule out a contribution from newly generated immature DCs.

For this reason, we wanted to be able to assess T cell activation within hours of antigen presentation by mature DCs. We therefore began our *in vivo* studies by assessing T cell activation by measuring CD69 up-regulation of adoptively transferred CD4+ OT.II T cells. CD69 is one of the earliest markers of T cell activation and is up-regulated within an hour after the T cell signaling is initiated (Craston et al., 1997). This way there would not be enough time for the repopulation of immature DCs and any T cell activation could be attributed to mature DCs.

Results

Reduced Presentation of Receptor-Targeted Antigen *In Vivo*

To induce systemic inflammation leading to the maturation of splenic DCs, we injected mice with 3 μg of LPS i.p. Control mice were injected with PBS. So that our results would be comparable to previous studies, we then waited 9 hours to allow for functional maturation before immunizing mice intravenously with soluble OVA or OVA immune complexes. T cell activation was assessed by measuring CD69 up-regulation of adoptively transferred CD4+ OT.II T cells.

In control mice that were injected with either soluble OVA or with immune complexes, we observed robust splenic T cell activation (Fig. 20, top row). In agreement with our *in vitro* data, there was poor T cell activation when soluble OVA was administered to mice that had previously been treated with LPS (Fig. 20, left column). However, we also found that there was poor presentation of receptor-targeted immune complexes under inflammatory conditions (Fig. 20, second column from left). Importantly, injection of peptide, which does not require internalization or processing, led to robust T cell activation (Fig. 20, third column from left). This shows that T cell activation itself is not impaired due to the systemic inflammation induced by LPS.

This data, which suggests that even receptor-targeted antigen is not presented efficiently by pre-matured DCs is inconsistent with our *in vitro* experiments in chapter 3. We felt that there were two general explanations that were most likely. The first is that splenic DCs behave differently than the BMDCs

that we used for most of our *in vitro* assays. The second is that mature DCs do no acquire antigens efficiently under conditions of systemic inflammation *in vivo*. This could be due to either poor uptake at the cellular level, or through systemic changes that reduce the concentration of antigens in the local environment.

Splenic DCs Efficiently Present Receptor-Targeted Antigens *In Vitro*

To determine whether splenic DCs behaved differently than BMDCs, we tested whether splenic DCs that were matured *in vivo* could present antigens delivered *in vitro*. As before, we matured DCs *in vivo* with LPS delivered i.p. or injected mice with PBS to leave the cells immature. Splenic DCs were harvested 9 hours later. We then incubated these cells with antigen and assessed their ability to present antigen to naïve T cells. Here we found that mature DCs presented both DEC-205-OVA and OVA-immune complexes at levels consistent with what we had observed with BMDCs (Fig. 21). Again, T cell activation was higher in mature DCs than immature DCs. However, unlike what we had observed with BMDCs, we found that mature splenic DCs presented soluble OVA comparably with immature DCs (Fig. 21). Regardless, it was unlikely that the reduced presentation by mature DCs *in vivo* was due to an inherent difference between splenic DCs and BMDCs.

We now determined that it was most likely that under the inflammatory conditions that we induced *in vivo*, mature splenic DCs had reduced access to antigens. To initially test this hypothesis, we conducted an experiment in which both the maturation stimulus and antigen were delivered *in vivo*, followed by an *in*

vitro assessment of antigen presentation. This way we could rule out the possibility that systemic inflammation somehow interferes with DC/T cell interactions *in vivo*. In these experiments, LPS or PBS controls were injected i.p. 9 hours before soluble OVA or OVA immune complexes were delivered i.v. DCs were harvested 9 hours later and cocultured with CFSE labeled T cells *in vitro*. As with the entirely *in vivo* assay, we witnessed poor presentation of antigen by mature DCs, regardless of the type of antigen used (Fig. 22). This experiment added support to our the hypothesis that lack of *in vivo* antigen presentation is most likely due to poor antigen acquisition.

Reduced Antigen Acquisition *in Vivo*

Our previous experiments, when taken together, strongly suggested that it was not an intrinsic inability of mature DCs to present antigen, but a lack of opportunity. We tested this hypothesis more directly by assessing the ability of i.v.-injected fluorescently labeled anti-DEC-205 antibodies to bind mature splenic DCs *in situ*. Binding of i.v.-injected antibody would require only that the antibody reach the cells: any modulation of endocytic activity should not alter the readout as it would with fluid phase tracers. Importantly, levels of DEC-205 in CD8α+ cells remain stable after maturation (Fig. 1B).

Mice were treated with LPS i.p. or with PBS control, as they had been in previous *in vivo* experiments. Fluorescently labeled anti-DEC-205 or isotype control antibody was injected i.v. Two hours later, spleens were harvested, and

DCs were analyzed by flow cytometry. Compared to immature splenic DCs, mature splenic DCs in LPS-treated mice showed greatly reduced labeling with the antibody (Fig. 23A). This result further suggests that systemic inflammation may limit access of splenic DCs to antigens.

Reduction of Splenic Blood Flow Under Inflammatory Conditions

The conditions that we and others have used to induce DC maturation *in vivo* are highly inflammatory, and may lead to a host of systemic changes, only one of which is the activation of the DC maturation program. After administration of TLR agonists, we had observed that the spleens change in nature, becoming darker and more friable. We asked if an alteration in something as simple as splenic perfusion itself could interfere with entry of antigen or even DC precursors, and at least partially explain the reduced access of mature DCs to antigens *in vivo*.

The development of microbubble contrast agents has allowed for the use of ultrasound technology detecting flow within microvessels as small as 7–10 μm. This technology allows for quantification of perfusion through the measurement of changes in signal intensity over time in a particular region of interest (Li et al., 2002; Schwarz et al., 1993). This technique has been used to monitor perfusion of tumors in murine cancer models (Fleischer et al., 2004), and we thought it could be a useful, non-invasive method of assessing splenic blood flow under inflammatory conditions.

Once again, we injected mice i.p. with LPS or PBS. Nine hours later, we administered microbubble contrast agent and assessed splenic perfusion via ultrasound. We observed a significant and reproducible (though incomplete) reduction in blood flow in the LPS-treated mice (Fig. 23B&C). This alteration may explain the failure of splenic DCs to access IV-injected antigen in our, and in previous studies.

Mature LN DCs Efficiently Present New Antigens

It is unclear how relevant this reduction of splenic DC access to antigen is under normal conditions. The i.v. injection of LPS most likely mimics sepsis, which is relatively rare though frequently fatal (Hotchkiss and Karl, 2003). However, the vast majority of infections are local, and immune responses are generated from interactions arising in draining LNs. Unlike the spleen, which acquires antigens through blood flow, the LNs receive input from afferent lymphatics. Antigens either drain from the periphery or are carried in by migratory DCs. We hypothesized that since blood flow was not involved in transporting antigens, there might be equal access of antigen to draining LNs even under circumstances such as systemic inflammation.

We therefore chose to examine the ability of LPS-matured DCs to access and present antigens in lymph nodes (LN). We found that while injection of LPS leads to full maturation of all LN DCs (Fig. 24A), access of antigen via lymphatics was not altered (not shown). We therefore chose to assess antigen presentation

within draining LNs *in vivo* using the previously established method of injecting Eα protein as a model antigen and assessing antigen presentation using the YAe antibody (Itano et al., 2003).

We again induced maturation with injection of LPS or PBS control i.p. Nine hours later, we injected Eα immune complexes or Eα aggregates s.c. in the hind hocks of the mice. Four hours later, we harvested the draining popliteal LNs and assessed antigen presentation using the YAe antibody. We found similar levels of presentation by immature DCs of the two antigen formulations on MHCII. However, consistent with our *in vitro* data, only Eα immune complexes were efficiently presented by LPS-matured LN DCs (Fig. 24B). These data indicate that fully mature LN DCs are able to capture and present receptor-targeted antigen *in vivo*.

Discussion

The relevance of features of the DC maturation program to the immune response *in vivo* has frequently been inferred from *in vitro* studies. Confirmation often proves difficult: taking the same reductionist approach *in vivo* is virtually impossible. While confirmation of the relevance of *in vitro* findings to physiologic systems is necessary, much care must be taken when interpreting results.

Two studies have recently correlated reduced phagocytosis and levels of MHCII synthesis in mature DCs with impaired immune responses to pathogens or model antigens (Wilson et al., 2006; Young et al., 2007). We found that in agreement with the latter report, splenic DCs do not present antigen introduced into the blood stream *in vivo* after the induction of systemic inflammation (Young et al., 2007). However, further investigation revealed that lack of presentation by splenic DCs is likely due to a reduction of access to antigens rather than an intrinsic failure of DCs (Fig. 23). Mature splenic DCs do present antigens efficiently if antigen is supplied *in vitro* (Fig. 21). Furthermore, antigen presentation occurs *in vivo* when cells have access to antigens, as is the case with LN DCs that acquire antigens after s.c. injection (Fig. 24B).

The major difference that we observed between BMDCs and splenic DCs *in vitro* was that presentation of untargeted OVA was not down-regulated by mature splenic DCs (Fig. 21). This persistence of fluid endocytosis may reflect the fact that splenic DCs that have been activated *in vivo* were harvested relatively soon after

LPS administration (at approximately 9 hours). In addition, splenic DCs down-regulate macropinocytosis with slower kinetics than BMDCs and exhibit residual fluid phase uptake for at least 24 hours (Garrett et al., 2000; West et al., 2004).

Our data supporting reduced antigen access of splenic DCs under inflammatory conditions further underscores the difficulty of attributing changes in complex systems to individual modifications. It is possible to add 50 ng of LPS to media within a tissue culture plate, and visualize the corresponding decline in endocytic rate that follows hours later. It is far different to inject a mouse with the same compound. The resulting systemic inflammation can lead to changes in body temperature, blood pressure, heart rate as well as the activation of many other cell types, that by their very nature are designed to interact and modulate one another (Cinel and Opal, 2009).

While to our knowledge, we are the first to report a reduction in splenic blood flow after injection of TLR ligands (Fig. 23 B&C), there are reports of inflammatory stimuli altering splenic organization and perfusion. It has been shown previously that blood flow to the spleen (Rogausch et al., 2003) and lymph nodes (Ottaway and Parrott, 1979; Soderberg et al., 2005) increases after the administration of an agent such LPS. This has been hypothesized to increase likelihood that a lymphocyte will find its match (Soderberg et al., 2005). Our study differed from the previous study of splenic perfusion in that we used 15-fold more LPS. While our dose of LPS is well within the range used in previous studies of DC maturation (Wilson et al., 2004) we witnessed dramatically different changes in

perfusion. The effect of LPS is dose-dependent and it is possible that the increased dose that we (and others) have used to assess DC maturation leads to systemic dysfunction.

Still, the reduction of blood flow was incomplete and therefore unlikely to account for the entire reduction in T cell activation. There are other physiologic changes that could also lead to reduced antigen access. One that is especially likely is DC redistribution within the spleen. Even though resident DCs continue to reside in the spleen after maturation, systemic LPS administration leads to a migration of CD11c+ DCs from the marginal zone of the spleen to the T cell area (De Smedt et al., 1996; Idoyaga et al., 2009). Additionally, injections of particulate antigen have been shown to accumulate in the marginal zone but not the T cell zone of the spleen (Drutman and Trombetta, in press). This adds support to our hypothesis that mature splenic DCs are exposed to reduced local antigen concentrations that explain reduced antigen presentation.

One piece of data contradicts this model. While splenic DCs apparently did not have access to i.v.-injected OVA, OVA ICs or anti-DEC-205 antibody, they did have access to i.v.-injected OVA peptides, which were presented with high efficiency, even under inflammatory conditions (Fig. 20). Although we do not have a definitive explanation for this finding, it is possible that small peptides have greater access to mature splenic DCs than larger, intact proteins. Moreover, peptides were delivered in amounts that led to dramatic CD69 elevation (Fig. 20).

It is possible that reduced levels of peptide on the mature splenic DCs were not detected due to saturation of the system.

Still, we do not wish to overstate the importance of reduced antigen access by mature splenic DCs. Its implications for understanding how to interpret past *in vivo* models of DC maturation are probably greater than any implications for understanding how the generation of immune responses proceed under physiologic conditions. Induction of systemic inflammation through introduction of micrograms of purified LPS or other TLR ligand into the bloodstream of an organism may mimic sepsis, but is unlikely to truly model how mature DCs function in the majority of circumstances. Since LN DCs are able to present antigens even under these conditions, we know that presentation of receptor-targeted antigen can occur *in vivo*, just as it does *in vitro*. Still, the frequency with which this happens, and the immunological implications, remain to be determined.

Materials and Methods

In Vivo Splenic DC Antigen Presentation Assays

$1x10^6$ CFSE labeled T cells were injected into the tail veins of mice. 24 h later mice were injected i.p. with 3 μg LPS or PBS control. After an additional 5 h, OVA-immune complexes or soluble OVA were injected i.v. Spleens were harvested and T cell activation was assessed by CD69 up-regulation of CFSE positive cells, 4 h after antigen injection. Alternatively, soluble OVA or DEC-205 OVA was injected subcutaneously into the hind leg hocks of OT.II mice 9 h after i.p. injection with 3 μg LPS or PBS. Popliteal LNs were harvested 4 h later and assessed by CD69 up-regulation of CD4+ T cells.

Ex Vivo Antigen Presentation Assay

DCs were incubated for 30 min with DEC-205-OVA, OVA immune complexes or soluble OVA in the presence of 50 μg/ml of LPS (Sigma). Cells were washed and the indicated number of DCs were cocultured $5x10^6$ CD4 or CD8 T cells that had been purified (Miltenyi) from the spleens of OT.II or OT.I mice respectively. 60 hours later CFSE labeled T cells were stained with anti-CD4 analyzed by flow cytometry to determine the extent of cell division. Immune complexes were made from polyclonal rabbit anti-OVA antibodies mixed at a 3:1 molar ratio with OVA (Worthington).

In Vivo LN DC Antigen Presentation Assay

25 μg of Eα aggregates or Eα immune complexes were injected into the hind leg hocks 9 hours after i.p. injection with 3 μg LPS or PBS control. Popliteal LNs were harvested 4 h later. 4 LNs from each condition were pooled so that there would be a sufficient number of cells for analysis. LNs were incubated in cell dissociation buffer (invitrogen) containing liberase blendzyme II and DNAse at 37° C for 20 min. Cells were labeled with YAe-biotin, washed, then labeled for a second time with anti-CD11c-APC, anti-CD3-Pacific Blue, anti-B220-Pacific blue. CD11c+ DCs were gated using FlowJo software and YAe intensity was presented as histograms

In Vitro Splenic DC Antigen Presentation Assay

Splenic DCs were obtained from the spleens of mice that had been injected IP with 3 μg of LPS (mature) or with PBS (immature). Tissue was cut into small pieces and incubated at 37° C with Liberase Blendzyme II and DNase (Invitrogen) before cells were dissociated by passage through a 70 μm cell strainer (BD). Cells were purified with CD11c+ cell isolation beads after harvest (Miltenyi).

DCs were incubated for 30 min with DEC-205-OVA, OVA immune complexes or soluble OVA in the presence of 50 μg/ml of LPS (Sigma). Cells were washed and the indicated number of DCs were cocultured $5x10^6$ CFSE-labeled CD4+ cells that had been purified using negative selection (Miltenyi) from the spleens of OT.II.

60 h later, T cells were stained with anti-CD4 antibody and were analyzed by flow cytometry to determine the extent of cell division. CD4+ T cells were gated and CFSE dilution was assessed. The percentage of dividing cells was determined for each sample. Anti-DEC-205-OVA was a gift from Ralph Steinman and was prepared from stably transfected lines of mouse myeloma cells using the LONZA recombinant protein expression system. Immune complexes were made from polyclonal rabbit anti-OVA antibodies mixed at a 3:1 molar ratio with OVA (Worthington). Soluble OVA (Worthington) was purified by centrifugal filtration (Millipore) or size-exclusion chromatography (Bio-Rad) to remove impurities and pre-formed peptides.

Flow Cytometry

Cells were resuspended in PBS containing 0.5% BSA, stained for 15 min on ice with antibodies conjugated with FITC, PE, PE-Cy7, pacific blue, PE-Cy5 or APC, then washed twice with buffer. The stained cells were fixed with 1% PFA, then subjected to FACS (BD Canto-II, FACSCalibur, or Aria). The collected data was analyzed with FlowJo software.

Antibodies

Anti-CD86-FITC/ PE/APC (GL1), anti-CD8a-PerCP (53-6.7), anti-CD45R-Pacific Blue (RA3-6B2), anti-CD11c-PE-Cy5 (HL3), anti-CD69-PE (H1.2F3), and streptavidin-PE were purchased from BD. Anti-CD3-Pacific Blue (17A2) and anti-

CD11c-Pacific Blue (N418) were purchased from BioLegend. Anti-6xHis was purchased from Sigma and Serotec. Anti-MHCII/Eα peptide (52-68)-biotin (YAe) was purchased from eBiosciences. Anti-OVA (Delamarre et al., 2003) is a rabbit polyclonal antibody.

Micro-ultrasound perfusion imaging

Mice anesthetized using 2% isoflurane delivered in medical air at 1 L/sec flow rate were placed supine on a dedicated small animal holding system (VisualSonics Inc., Toronto, ON, Canada). Body temperature and heart rate were monitored for the remainder of the procedure (THM 150, Indus Instruments, Houston, TX, USA). Temperature was maintained at 37° C with a heated imaging platform. Imaging was performed with a Vevo 770 micro-imaging system (VisualSonics Inc., Toronto, Ontario, Canada) employing a single-element ultrasound transducer with a 40 MHz center frequency and a focal length of 6 mm. A micro-bubble ultrasound contrast agent (Definity®, Bristol-Meyers Squibb Medical Imaging, Inc., Billerica, MA, USA) was administered as an intravenous bolus injection of 10 µl through a jugular vein puncture. Single-slice B mode imaging was performed (center frequency = 40 MHz, 50% power, axial resolution = 40 µm, lateral resolution = 100 µm, 20 frames per second). The ultrasound probe was aligned perpendicular to the animal and the same anatomical spleen slice was determined for each animal. 800 frames of ultrasound data were acquired for first-pass kinetics analysis, the first 20 of which were acquired prior to contrast agent injection. First-pass kinetics

analysis of the signal intensity-time curve was employed to measure relative blood volume (BV) and vascular transit time (VTT) for each pixel (1, 2). Relative blood flow (rBF) was obtained for each pixel from the ratio of rBV to the VTT.

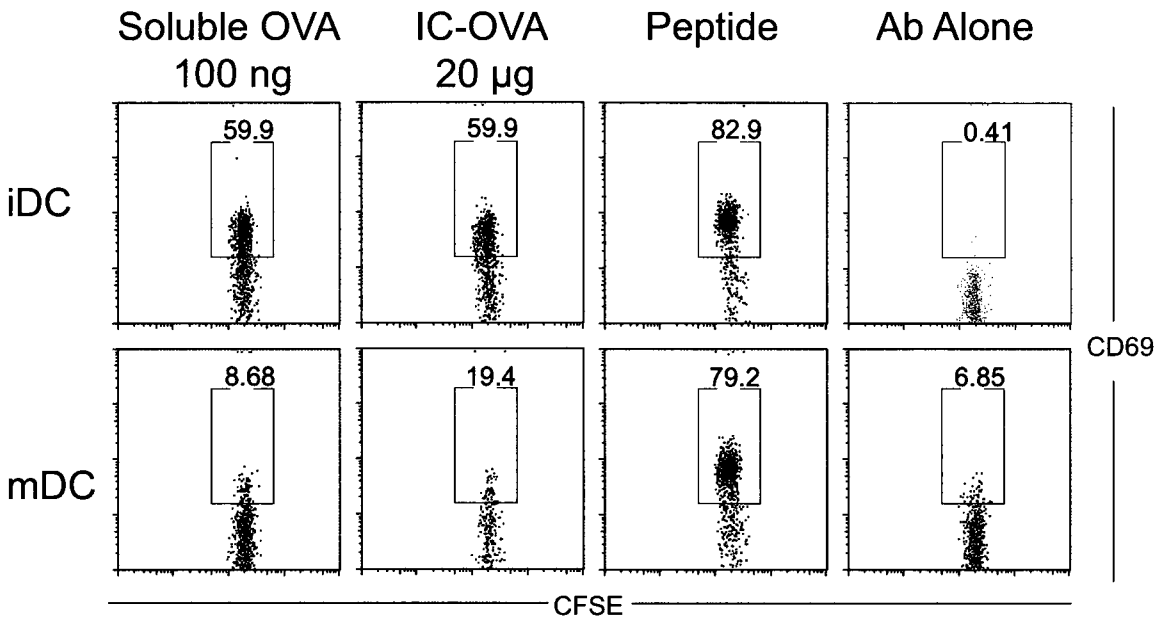

Figure 20 - Systemic inflammation leads to decreased activation of T cells *in vivo*. 10e6 CFSE-labeled OT.II CD4+ T cells were injected i.v. 24 h later mice were injected i.p. with 3 µg LPS or PBS control. After 5 additional h, OVA immune complexes or soluble OVA were injected i.v. 4 h later, spleens were harvested and T cell activation was assessed by CD69 upregulation.

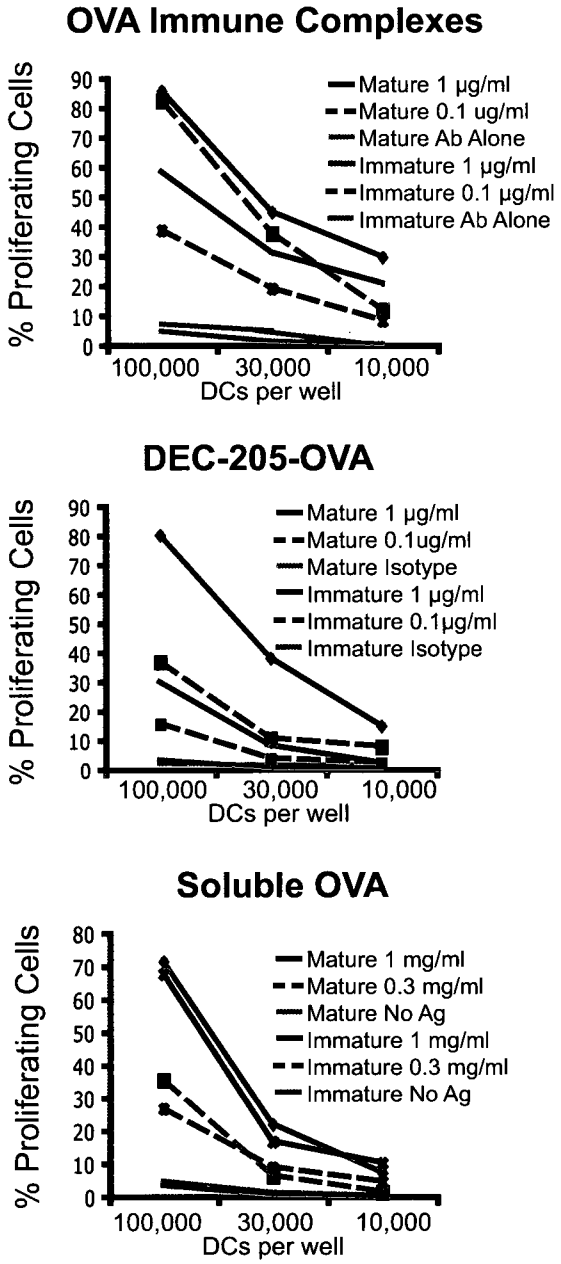

Figure 21 - Mature splenic DCs present antigen *in vitro*.
Splenic DCs were isolated from mice injected with 3 µg LPS or PBS i.p. DCs were then incubated with anti-DEC-205-OVA, OVA immune complexes or soluble OVA at the indicated doses for 2 h before the cells were washed and co-cultured with CFSE-labeled CD4+ OT.II T cells. 60 h later the extent of cell division was determined by flow cytometry. Experiment performed in duplicate. Average values are displayed.

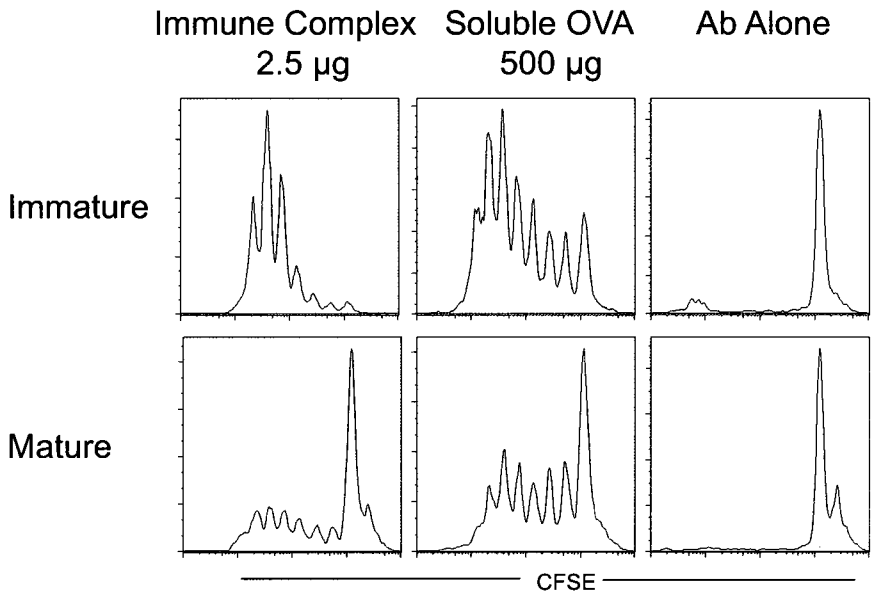

Figure 22- Reduced presentation of antigen by mature splenic DCs *ex vivo*. Mice were injected i.p. with 3 µg LPS or PBS control. 9 h later, OVA-ICs, soluble OVA, rabbit anti-OVA antibody as control were injected i.v. Spleens were harvested 2 h later and DCs were isolated and cultured with CFSE-labeled CD4+ OT.II T cells. 60 h later the extent of cell division was determined by flow cytometry. A representative experiment is displayed.

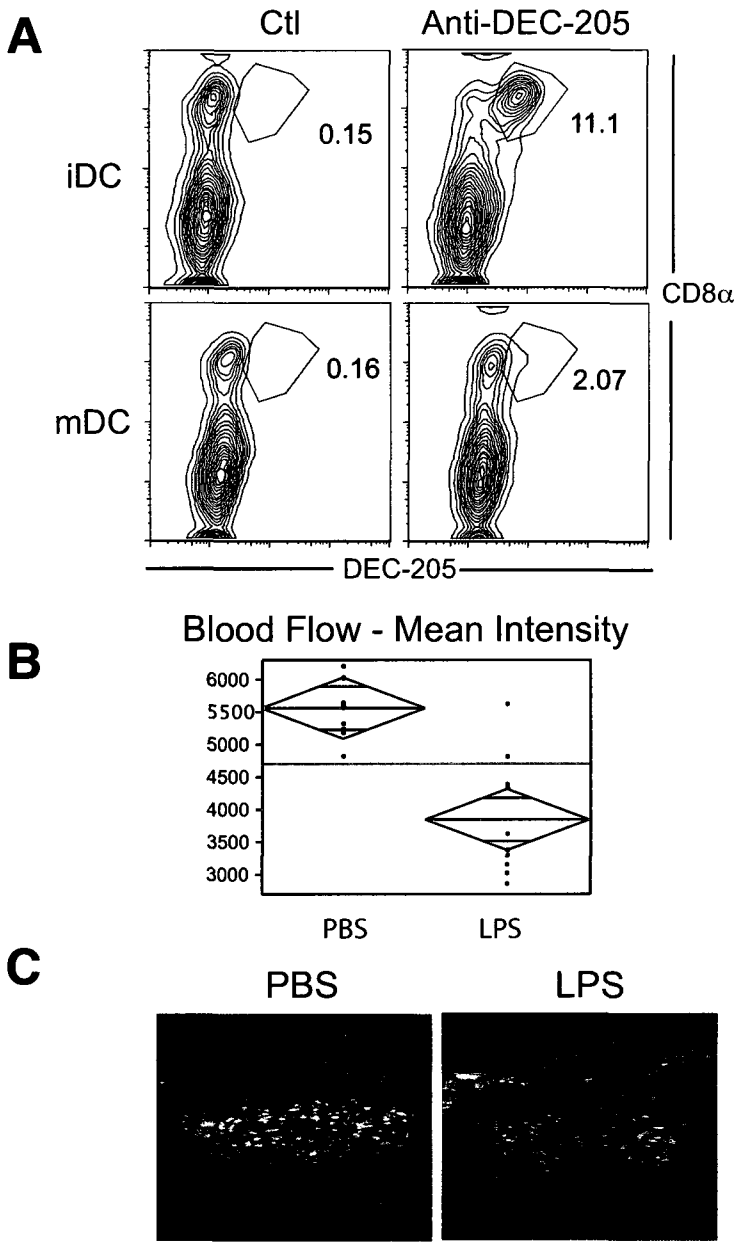

Figure 23 - Systemic administration of LPS reduces access of splenic DCs to antigen.

(A) 3 μg anti-DEC-205-FITC or isotype control antibody was injected i.v. into mice that had been pretreated for 9 h with 3 μg LPS i.p. or PBS control. 2 h later the spleens were harvested and DCs were analyzed by flow cytometry. Histograms of CD11c+ cells are displayed.

(B) Mice were injected i.p. with 3 μg LPS or PBS control. 9 h later mice were anesthetized and administered 50 μl of microbubbles via the jugular vein. Splenic perfusion was monitored by ultrasound. Results from 10 mice from each group are displayed.

(C) Representative ultrasound images obtained during procedure outlined in (B).

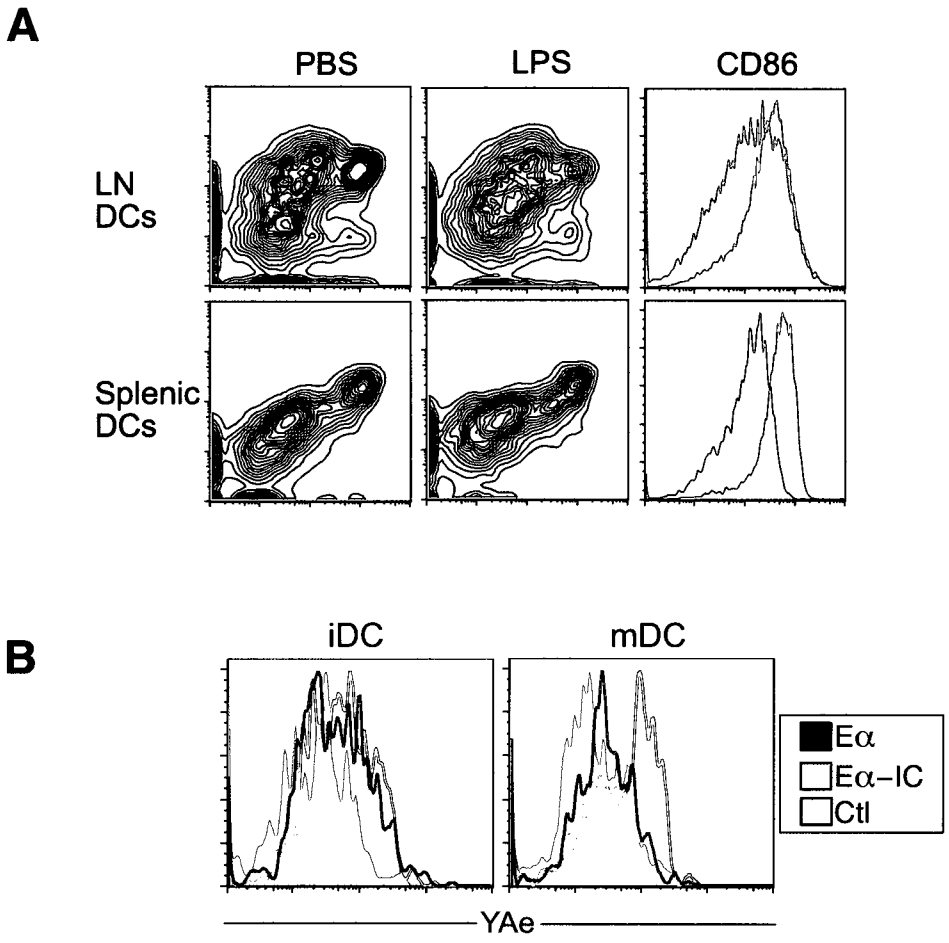

Figure 24 - Mature LN DCs present receptor targeted antigen *in vivo*.
(A) Splenic or LN DCs were harvested from LPS treated (3 μg i.p.) or PBS treated control mice 9 h after injection. Cells were dissociated, labeled with anti-DEC-205, anti-CD8α, anti-CD11c and anti-CD86 and analyzed by flow cytometry. Plots are gated on the CD11c+ population.
(B) Mice were injected with 3 μg LPS i.p. or PBS control. 9 h later, Eα aggregates, Eα aggregate ICs or PBS as control were injected subcutaneously into the hind leg hocks of the mice (each injection contained 1 μg LPS). 4 h later, the draining popliteal lymph nodes were harvested. Histograms are of CD11c+ cells.

Chapter 6: Conclusions and Future Directions

Mature DCs continue to have the ability to take up and present antigens, both *in vitro* and *in vivo*. This statement should be viewed as a refinement, rather than an upheaval of the dogma concerning DC maturation. Our finding that endocytic receptors remain completely functional even as macropinocytosis is markedly down-regulated demonstrates that DCs are not developmentally restricted to acquiring antigens during their immature state. Like many new findings, it will likely be some time before the implications are clear.

Maturation Leads to Increased Antigen Selectivity

We propose a model in which part of the DC maturation program is to become more selective of antigen uptake. It is well established that during maturation, non-specific forms of endocytosis, macropinocytosis and non-receptor-mediated phagocytosis are down-regulated (Garrett et al., 2000; Reis e Sousa et al., 1993; Sallusto et al., 1995). Since receptor-mediated uptake remains intact (Fig. 10, 12, 14 & 24), uptake is targeted to antigens that have already been recognized by the immune system (and are complexed with specific IgG) or that bind to specific pathogen receptors (eg lectins) on the DC surface. Self-antigens may not have comparable receptors and therefore would be less likely to gain entry under these conditions. Thus our findings need not immediately discount any role that modulation of antigen uptake plays in preventing presentation of self-antigens.

A better understanding of the various ways in which antigen is encountered and internalized in a physiologic setting will allow us to determine the importance of antigen acquisition by already mature DCs. When a larger number of DC antigen receptors and known ligands are compiled, it will be possible to determine whether receptor modulation upon maturation corresponds with the nature of the ligand. For instance, one might expect that DCs matured by PAMPs would down-regulate receptors that take up primarily self-antigens while up-regulating receptors that target foreign antigens. Differential receptor expression by spontaneously matured or tolerogenic DCs has not been documented but might be expected if this model were accurate.

However, as our understanding of the immune system becomes increasingly nuanced, the notion that the down-regulation of macropinocytosis or specific endocytic receptors is essential to maintaining tolerance seems less likely. When an immature DC encounters a pathogenic stimulus, the DC has undoubtedly already internalized substantial quantities of self-antigen. And while the signal induced by PAMP recognition indicates the presence of a pathogenic threat (Pasare and Medzhitov, 2005), it certainly does not indicate the absence of self-antigen. Indeed, phagocytosis of a bacterium does not preclude uptake of self in another phagosome.

Several mechanisms are known that ensure that the immune response is properly focused on pathogens. Phagosome maturation has been shown to progress autonomously in a TLR-dependent manner (Blander and Medzhitov,

2004). This leads to PAMP-associated cargo being presented more efficiently than non-PAMP associated cargo within the same cell (Blander and Medzhitov, 2006b). Thus, subcellular mechanisms exist to ensure that antigen presentation is biased towards foreign threats, even when such cargo is internalized concurrently with self-antigen. If mature DCs were to continue to indiscriminately internalize both PAMP- and non-PAMP-associated cargo via endocytic receptors, these cells could presumably avoid presentation of self.

However, there are circumstances where neither downmodulation of antigen uptake nor phagosome-autonomous antigen processing can explain the lack of presentation of self-antigens by immunogenic DCs. One especially problematic example would be virally infected apoptotic cells: they contain a mix of foreign and self (Blander and Medzhitov, 2006a). It is under these circumstances that it is likely that one of the many safety nets is employed. Central tolerance plays a vital role in preventing autoimmunity by leading to the deletion of autoreactive T cells before they can be released into the periphery for DC activation (Mathis and Benoist, 2007). Even when such autoreactive T cells escape, mechanisms of peripheral tolerance, such as those mediated by regulatory T cells can prevent pathology (Yamazaki et al., 2008).

Implications for Timing of Antigen Acquisition

Our findings challenge the long-standing views that DCs can only present antigens encountered at the time of capture and initial induction of maturation

(Mellman and Steinman, 2001; Villadangos et al., 2005). Since mature DCs appear capable of specific antigen uptake, processing, and presentation, the DC system would remain available to initiate T cell responses against new or mutating microbes and viruses during the course of an infection when many DCs are already mature.

This forces us to ask the additional question of whether mature DCs can be "reprogrammed" to induce a qualitatively different type of immune response than that induced by the initial maturation stimulus, e.g. by altering the profile of secreted cytokines. We think that this is likely to be the case because spontaneously-matured tolerogenic DCs can be induced to produce inflammatory cytokines by supplying a later TLR stimulus (Jiang et al., 2007). If such reprogramming can occur, the cytokines released during antigen presentation would be related to their more recent stimulus, not their original stimulus. This might be detrimental if two concurrent infections required different responses. Further research will be required to determine how DCs provide the proper instructions to T cells in such situations.

Implications for Different Modes of Maturation

Our study focused exclusively on maturation induced by TLR ligands, primarily LPS. While this is a highly physiologic means of DC maturation, it will be important to examine other forms. In the earliest studies of DCs, maturation was inadvertently induced by dissociation from lymphatic tissue, a necessary step in

obtaining populations of cells for study (Reis e Sousa et al., 1993; Steinman and Cohn, 1973; Steinman and Witmer, 1978). Many assumptions that we make about mature DCs are still inferred from studies of these cells. Now we know that maturation can be induced by a host of microbial products, lymphocytes via CD40 and lymphotoxin αβ receptors, cytokines, endogenous mediators such as uric acid and heat-shock proteins, immune complexes, and disruption of cell-cell contacts (Jiang et al., 2007; Steinman and Banchereau, 2007).

The way in which maturation is induced influences the type of immune response generated. Unlike BMDCs matured by TLR ligands, spontaneously matured BMDCs have an anti-inflammatory gene expression profile and have been determined to induce tolerance in the EAE model (Jiang et al., 2007). On the other hand, like TLR-induced maturation, CD40-initiated signaling is sufficient for the induction of effective immune responses (Hawiger et al., 2001). However, one study showed that while CD40-matured DCs presented phagocytosed antigens, LPS-matured DCs did not (Nayak et al., 2006). Clearly, differences in antigen presentation and cytokine production by differentially matured DCs need to be understood in greater detail.

As mentioned earlier, DCs can be activated, *in vivo,* indirectly by inflammatory cytokines such as IFNα, IFNβ, TNFα and IL-2. PAMP recognition by both immune and non-immune cells can lead to cytokine production to which DCs respond by up-regulation of both MHC molecules and costimulatory molecules. Interestingly, these DCs lack the ability to drive differentiation of naive CD4+ T

cells into effector T cells *in vivo* (Sporri and Reis e Sousa, 2005). It makes sense that these DCs should not lead to T cell activation: such indirect activation would be counter-productive if DCs were not presenting pathogen-derived antigens. However, it would clearly be harmful if indirect activation put the host's entire network of DCs effectively out of commission before they had the opportunity to access antigens. It would therefore be especially useful if DCs matured indirectly were able to continue to present antigens, especially if they could be "reprogrammed" by subsequent contact with PAMP associated antigens.

In Vivo Significance

Experimental models of DC maturation have involved the injection of TLR agonists. This induces other physiological changes that influence the ability of DCs to encounter antigens. It is essential to remember even as we dissect how cells function on an individual level that they are components of a complex larger system.

We will not be able to determine the *in vivo* significance of our findings without dissecting how these findings apply to the various DC subsets. We expect that our study is likely to be broadly applicable because we have demonstrated that mature DCs can present antigen whether the source of the DCs was spleen, LN or bone marrow culture. However, we have not examined any of the migratory DC subsets. One might expect that these DCs would be more resistant to taking up and presenting new antigens since their role seems uniquely suited to presenting

antigens from their destination. Interestingly, this does not seem to be the case. One DC subset that was previously reported to present new antigens *in vivo* (but not *in vitro*) is the prototypically migratory Langerhans cell (Ruedl et al., 2001).

Despite the advances made in the cell biology of antigen presentation, we still have a fairly limited understanding of how antigen is taken up *in vivo*. Most studies that have tested the ability of mature and immature DCs to capture and present antigen have been done with soluble antigens that are taken up by macropinocytosis. The fact that *in vitro* studies have for so long focused on this form of uptake likely overstates its importance. Presentation of antigens taken up via this route is extremely inefficient compared to presentation of immune complexes or anti-DEC-205-OVA. Previous work has demonstrated that antigens targeted to these receptors are presented on MHCI and MHCII over 100 fold more efficiently than antigens taken up non-specifically (Bonifaz et al., 2004; Regnault et al., 1999). These antigen concentrations are far more likely to be physiologic than the milligrams per milliliter of soluble antigen necessary for macropinocytosis-mediated antigen presentation.

Unfortunately, it is extremely difficult to examine antigen uptake and presentation *in situ*. Attempts to assess this are invariably complicated by the application of agents that induce systemic inflammation. However, the use of intravital microscopy has allowed visualization of the intricacies of T cell priming (Mempel et al., 2004) and even antigen sampling (Chieppa et al., 2006) by DCs

under physiologic conditions. Such technology could also be applied to assess uptake and presentation of antigen by both immature and mature DCs *in vivo*.

Clinical Significance

The simplified view of DCs as either mediators of immunity or tolerance glosses over the varied types of immune responses that have been demonstrated (Steinman and Banchereau, 2007). The roles that functionally different DCs play in myriad clinical situations are even more wide-ranging. DCs have been implicated in playing roles in infection, cancer, allergy, autoimmunity, chronic inflammation, transplantation and vaccination (Steinman and Banchereau, 2007). As DC maturation is responsible for both optimizing antigen presentation and determining the nature of the immune response, an enhanced understanding of DC maturation will no doubt play a role in how we approach DCs in our quest to maintain health and prevent disease.

Presumably, the benefit of antigen uptake and processing that continues hours after maturation outweighs any potential harm. However, the immune system must constantly perform a balancing act between immunity and tolerance. Going too far in either direction results in disease (Cools et al., 2007). Like any important process, antigen presentation has the potential for dysregulation and it is certainly possible that in some cases disease is mediated by mature DCs presenting antigen inappropriately.

In our study we show that the FcγR mediates presentation of immune complexes in already mature DCs. The basic function of immune complex clearance is thought to be a vital part of immune homeostasis, with dysregulation leading to autoimmune disease such as systemic lupus erythematosus (SLE) (Takai, 2005). It is conceivable that capture of self antigen-antibody complexes by mature DCs during an infection could be involved in the initiation or exacerbation of autoimmune disorders associated with autoantibodies such as SLE. The presence of FcRs on DCs have also been shown to be critical to development of inflammation associated with hyperresponsiveness to certain antigens (Kitamura et al., 2007).

A role for mature DCs presenting malignancy-related antigens is certainly possible as well. While the importance of immunosurveillance in preventing and controlling the development of malignancies is not entirely settled, there a plethora of evidence of anti-cancer immune responses (Dunn et al., 2004). T-cell infiltrates have been detected within a variety of primary tumors and this has been associated with dramatically improved prognosis (Galon et al., 2006; Zhang et al., 2003). Tumor-infiltrating DCs have been reported as well and interestingly, the maturation state of these DCs has also proven important to prognosis. Patients with intratumoral DCs that have a mature phenotype have better outcomes than patients with tumors containing DCs that appear immature DCs (Chaput et al., 2008). This may be attributed to basic tenets of DC biology: immature DCs are likely not presenting tumor antigens efficiently. Still we now know that it would be possible

for mature DCs to promote an anti-tumor response by continuing to acquire antigen even while in a mature state.

Conclusion

Our understanding of DC biology is growing increasingly complex. While we once thought of a prototypical DC, we now know of multiple subsets. We now know of increasingly numerous stimuli capable of inducing DC maturation and increasingly nuanced immune responses. In the preceding pages, we presented an additional layer of complexity to the DC system. The future will undoubtedly bring us a better understanding of how these layers of complexity interact. This should allow us to harness the power of the immune system to defeat infectious disease and malignancy or assuage the symptoms of allergy and inflammatory disease.

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