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Antigen presentation : Influence of Cell Type and Route of Antigen Uptake

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ANTIGEN PRESENTATION:

INFLUENCE OF CELL TYPE

AND ROUTE OF ANTIGEN UPTAKE

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Dendritic cells (DCs), which maintain tolerance and orchestrate T cell immune responses, comprise a heterogeneous group of cells. For example, in the steady state, murine spleen contains pre-DC-derived $CD8^{+}DEC\text{-}205^{+}$ and $CD8^{-}DCIR2^{+}$ conventional DCs. To examine antigen processing and presentation *in vivo*, antigens were specifically targeted to $CD8^{+}$ and $CD8^{-}$ DCs using chimeric monoclonal antibodies. We find that $CD8^{-}$ DCs are better than $CD8^{-}$ DCs for presentation of exogenous antigens onto major histocompatibility complex (MHC) class II molecules due to cell intrinsic differences.

DCs are responsible for initiating T cell responses. However, during inflammation, monocytes become activated and acquire some DC-like features such as expression of CD11c, MHCII and co-stimulatory molecules, yet their role in T cell activation is still a matter of investigation. Cells similar to DCs can also be produced *in vitro* by culturing monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) or bone marrow progenitors (pre-DCs) with fms-related tyrosine kinase ligand (FL). Although each of these cell types can present antigen, the relative efficiency of processing and

presentation after antigen capture by different routes has not yet been systematically compared. To this end we administered OVA to various conventional DCs and activated monocytes by receptor-mediated endocytosis, pinocytosis or phagocytosis and measured antigen uptake and presentation to MHCI and MHCII restricted T cells. We find that $CD8^-$ DCs are more efficient than any other types of antigen presenting cells tested in terms of presenting antigen to MHCII restricted $CD4^+$ T cells, irrespective of the route of antigen capture or maturation status. In contrast, both subsets of splenic DCs are equally efficient at cross-presenting antigens to $CD8^+$ T cells. All DCs and activated monocytes cross-presented antigens delivered by receptor-mediated endocytosis and pinocytosis, albeit with different efficiencies. However monocyte-derived cells differ from DCs in that they are several orders of magnitude less efficient in presenting antigens captured by phagocytosis. DCs derived from pre-DCs are unique, processing and presenting antigens efficiently irrespective of the route of antigen capture. Our observations have significant implications for understating initiation of immune responses and vaccination strategies targeting DCs and activated monocytes.

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List of Abbreviations

APC: antigen presenting cell

BCR: B cell receptor

BSA: bovine serum albumin

cDC: conventional dendritic cell

°C: degree Celsius

CD: cluster of differentiation

CDP: common dendritic cell progenitor

CS: circumsporozoite protein

CSf: circumsporozoite protein from *Plasmodium falciparum*

CSpep: peptide SYVPSAEQI from *Plasmodium yoelii* circumporozoite protein

CD11c-hDEC: CD11c promoter driving expression of hDEC

CFA: complete Freund's adjuvant

CFSE: 5-(6)-carboxyfluorescein diacetate succinimidyl diester

CHO: chinese hamster ovary

CLIP: Ii derived MHC class II-associated invariant chain peptide

Cy5: cyanine 5

DC: dendritic cell

EDTA: ethylenediaminetetraacetic acid

EEA1: early endosome antigen 1

ERAP : endoplasmic reticulum amino peptidases

ER: endoplasmic reticulum

Fc: fragment crystallizable region

FCS: fetal calf serum

FITC: fluorescein isothiocyanate

FL: fms-related tyrosine kinase ligand

FL-DCs: DCs differentiated from progenitors by culture with FL

GC: germinal center

GFP: green fluorescent protein

GM-DCs: GM-CSF monocyte-derived DCs

GM-CSF: granulocyte-macrophage colony-stimulating factor

HBSS: Hanks balanced salt solution

hDEC: human DEC-205

HEL: hen egg lysozyme

HRP: horseradish peroxidase

iCD8$^+$ DC: CD8$^+$ DC isolated from mice immunized with antigen in CFA and challenged with antigen 24 hrs before

iCD8$^-$ DC: CD8$^-$ DC isolated from mice immunized with antigen in CFA and challenged with antigen 24 hrs before

Ig: immunoglobulin

IL: interleukin

iMono: activated monocytes isolated from mice immunized with antigen in CFA and challenged with antigen 24 hrs before

iNOS: inducible nitric oxide synthase

i.p.: intraperiontoneal

i.v.: intravenous

KLH: keyhole limpet hemocyanin

LPS: lipopolysaccharides

mBSA: methylated BSA

M-CSF: macrophage colony stimulating factor

M-CSF-R: macrophage colony stimulating factor receptor (CD115)

mDEC: mouse DEC-205

MDP: macrophage and dendritic cell progenitor

MHC: major histocompatibility complex

MHCI: MHC class I

MHCII: MHC class II

MMR: macrophage mannose receptor

Mono: monocyte

MyD88: myeloid differentiation primary response gene 88

NOD: nucleotide oligomerization domain

NP: 4-hydroxy-3-nitrophenil

OVA: ovalbumin

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phycoerythrin

PerCP: peridinin chlorophyll a protein

pDC: plasmacytoid dendritic cell

PVDF: Polyvinylidene fluoride

Rcf: relative centrifugal force

RIG: retinoic acid inducible gene

Rpm: revolutions per minute

SE: standard error

TCR: T cell receptor

Tip-DC: TNF/iNOS producing dendritic cells

TLR: Toll like receptor

TNF: tumor necrosis factor

TRIF: TIR domain-containing adapter-inducing interferon-β

TSLP: thymic stromal lymphopoietin

WT: wild type

Chapter 1

Introduction

Dendritic Cells (DCs) are professional antigen presenting cells, necessary for maintaining tolerance and initiating immune responses. The primary function of DCs is to present antigens to T cells, while controlling and modulating T cell activation with cytokines and co-stimulatory molecules. DCs are very efficient at antigen uptake: they continuously internalize soluble antigens and express numerous receptors that bind and internalize pathogens. Captured antigens are processed into peptides for both MHC class II (MHCII) and MHC class I (MHCI) presentation (cross-presentation). Different subsets of DCs have been identified, but until recently, few functional differences between these subsets were defined.

In this thesis, I compare the antigen presentation efficiency of different DC subsets (splenic $CD8^+$ and $CD8^-$ DCs) for both MHCII presentation and cross-presentation. *In vivo* experiments were performed targeting antigen to DC receptors. These studies were supplemented with *ex-vivo* antigen administration through receptor-mediated endocytosis, phagocytosis and pinocytosis. I also analyzed the antigen presentation efficiency of activated monocytes that appear during inflammation. Furthermore, I compared splenic DCs to other antigen presenting cells (APCs) that can be differentiated *in vitro*. For the several different APCs analyzed, I address the impact of the route of antigen uptake and of cell intrinsic factors on antigen presentation efficiency.

Historical Perspective of Antigen Presentation

Unlike B cells, T cells require accessory cells for antigen recognition and activation (Waldron et al., 1973). Seminal studies in 1974 showed that to recognize antigen, T cells must share the same major histocompatibility complex (MHC) as accessory cells (Zinkernagel and Doherty, 1974).

The need for antigen processing by accessory cells was proposed because interactions between accessory cells and T cells only began 20-60 minutes after antigen internalization. During this processing time, active metabolism but not protein synthesis was necessary (Ziegler and Unanue, 1981).

A few years later, it was demonstrated that peptides are generated during processing, and bind to MHC molecules, allowing T cells to recognize antigen and become activated (Babbitt et al., 1985; Shimonkevitz et al., 1983). By the mid 1980's, the general principals of antigen processing and presentation for T cell activation were in place. Since then, many fundamental studies added to the complexity of this process, but many nuances and mysteries still remain unresolved.

Dendritic Cells

DCs were first recognized for their unique dendritic morphology (Steinman and Cohn, 1973). A few years later, DCs were characterized as potent stimulators of the mixed leukocyte reaction (Steinman and Witmer, 1978) and effector T cell responses (Nussenzweig et al., 1980; Steinman, 2007), orders of magnitude better than macrophages, the best accessory cells known at that time.

DCs can be found in lymphoid and non-lymphoid tissues. Although no single unique feature can distinguish all DCs, they are characterized by expression of CD11c (integrin αX) and high levels of MHCII. DCs consist of a heterogeneous population that can be divided into several subsets. Several aspects concerning subset grouping and defined functional specializations remain unresolved (Heath et al., 2004). Yet, recent data from ontogeny and gene expression support the current division of DC subsets in lymphoid organs (Edelson et al., 2010; Liu et al., 2009; Robbins et al., 2008).

Like all other immune cells, DCs originate from hematopoietic stem cells. They have a short half-life: spleen and lymph node DCs are replaced by progenitors from the blood within 10 to 14 days (Liu et al., 2007). Although DCs are closely related to monocytes and macrophages, they develop from committed progenitors that diverge in the bone marrow (Liu et al., 2009). The lineage split occurs when macrophage-and-DC progenitors (MDPs) (Fogg et al., 2006; Varol et al., 2007) differentiate into either monocytes or common-DC progenitors (CDPs) (Naik et al., 2007; Onai et al., 2007). CDPs no longer give rise to monocytes and macrophages, and they further differentiate into pre-DCs,

losing the potential to produce plasmacytoid DCs (Liu et al., 2009). Pre-DCs leave the bone marrow, circulate in the blood and seed lymphoid (Liu et al., 2009) and non-lymphoid organs (Bogunovic et al., 2009; Ginhoux et al., 2009; Varol et al., 2009). DCs divide in response to fms-related tyrosine kinase ligand (FL) (Liu et al., 2007; Waskow et al., 2008) and differentiate into subsets of conventional DCs. In lymphoid organs, DCs are incorporated into networks of cells and present antigen to migrating T cells (Lindquist et al., 2004; Liu et al., 2009).

Plasmacytoid dendritic cells (pDCs) are very distinct from other DCs, known as conventional DCs (cDCs). pDCs are $B220^{+}$, $CD11c^{int}$ and produce large amounts of type I interferon upon CpG DNA or viral exposure (Gilliet et al., 2008; Liu, 2005). pDCs are long-lived, finish their developmental program in the bone marrow, and do not show a dendritic-like morphology. Their role in antigen presentation is still debated (Bar-On and Jung, 2010), and pDCs will not be discussed further in this thesis.

In murine spleen, conventional DCs can be divided into two major subsets based on the expression of CD8α. The $CD8^{+}$ subset is also positive for DEC-205. The $CD8^{-}$ DC subset includes a major $CD4^{+}$ ($CD11b^{+}$ and $DCIR2^{+}$) population and a minor $CD4^{-}$ (DN, double negative) population. $CD8^{-}$ DCs localize in the marginal zone and the red pulp of the spleen, while $CD8^{+}$ DCs reside in the T cell area (Ardavin, 2003; Dudziak et al., 2007). In C57BL/6 mice the $DCIR2^{+}$ population represents more than 50% of splenic DCs. Both cDC subsets, $CD8^{+}$ and $CD8^{-}$, derive directly from pre-DCs and are usually called resident DCs (Liu et al., 2009).

In addition to resident DCs, lymph nodes also contain $CD11c^{int}$ and $MHCII^{hi}$ migratory DCs (Henri et al., 2001). DCs migrate from tissues to lymph nodes via lymphatics in a CCR7-dependent manner (Ohl et al., 2004). Migratory DCs can be further divided into different subsets. For example, the subsets can be based on the expression of CD11b, CD207 (langerin) and CD103 (integrin αE) (Henri et al., 2010; Nagao et al., 2009). Lymph nodes contain different populations of migratory DCs depending on the tissue drained.

The division of non-lymphoid organ DCs into subsets is more complex and only recently supported by functional and developmental data. Increasing evidence support a correspondence between $CD11b^{-}CD103^{+}$ DCs in non-lymphoid organs and $CD8^{+}$ DCs in lymphoid organs. For example, both $CD11b^{-}CD103^{+}$ dermal DCs and $CD8^{+}$ DCs excel in cross-presentation of cell-associated antigens (Bedoui et al., 2009) and are developmentally dependent on the basic leucine zipper transcription factor, ATF-like 3 (Batf3) (Hildner et al., 2008). Likewise, $CD11b^{-}CD103^{+}$ DCs in kidneys, lung and liver rely on the same cytokine (FL) and transcription factors (Id2 and IRF8) as $CD8^{+}$ splenic DCs (Ginhoux et al., 2009). However, unlike splenic DCs, tissue-resident $CD11b^{+}CD103^{-}$ DCs have a partial developmental dependence on M-CSF-R (macrophage colony stimulating factor receptor). Furthermore, although pre-DCs can give rise to both $CD11b^{+}CD103^{-}$ and $CD11b^{-}CD103^{+}$ DCs in liver and kidneys, monocytes can also differentiate into $CD11b^{+}CD103^{-}$ DCs. Therefore, $CD11b^{+}CD103^{-}$ DCs might constitute a heterogeneous population (Ginhoux et al., 2009).

The gut lamina propria contains pre-DC-derived $CD11b^{+}CD103^{+}$ DCs that support the development of regulatory T cells, and also monocyte-derived $CD11c^{+}CD103^{-}$ CX_3CR1^{+} cells that have a prominent role during inflammation (Bogunovic et al., 2009; Varol et al., 2009). Interestingly, lamina propria $CD11b^{+}$ $CD103^{+}$ DCs differ from $CD8^{+}$ splenic DCs and $CD11b^{-}CD103^{+}$ DCs found in Peyer's patches, in that the former are not dependent on IRF8 (Ginhoux et al., 2009) and Batf3 (Edelson et al., 2010).

During inflammation or infection, the distribution of DCs in lymphoid organs changes, and new subsets can appear (Serbina et al., 2008). Under these conditions, monocytes can differentiate into cells with DC-like features such as CD11c and MHCII expression (Geissmann et al., 2003; Leon et al., 2007; Naik et al., 2006; Randolph et al., 1999). For example, during *Listeria* infection monocytes differentiate in the spleen into tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS) producing $CD11c^{+}$ cells (tip-DCs) (Serbina et al., 2003). Nonetheless a prominent role of monocyte-derived DC-like cells in antigen presentation has not been established, and instead innate functions have been suggested (Serbina et al., 2003).

In Vitro Differentiation of Dendritic Cells from Precursors

Cells with many DC features can be derived from monocytes cultured with cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Inaba et al., 1992a; Sallusto and Lanzavecchia, 1994). GM-CSF was initially found to promote the survival of murine Langerhans cells (Witmer-Pack et al., 1987) and later shown to promote the development of $CD11c^+$ cells from blood (Inaba et al., 1992b) and bone marrow progenitors (Inaba et al., 1992a).

GM-CSF monocyte-derived DCs (GM-DCs) are widely used as a dendritic cell source, both in basic murine studies (Mellman and Steinman, 2001; Trombetta and Mellman, 2005) and in human clinical studies (Steinman and Dhodapkar, 2001). However, it was recently established that in steady state most conventional DCs are GM-CSF-independent, FL-dependent and derived from pre-DCs (Liu et al., 2009; Waskow et al., 2008). Also, GM-DCs seem to be more related to activated monocytes as measured by production of inflammatory mediators, such as TNF-α and nitric oxide (Xu et al., 2007), and global gene expression profile (Robbins et al., 2008).

GM-CSF deficient mice have normal hematopoietic development except for functional defects on alveolar macrophages (Stanley et al., 1994) and a lack of $CD103^+$ dermal DCs (King et al., 2010). GM-CSF administration in mice results in increased circulating neutrophils and cycling of peritoneal macrophages but no overt changes in DC numbers. In humans, GM-CSF administration increases blood monocyte numbers (Hamilton, 2002).

On the other hand, FL administration induces a dramatic increase in DC numbers in mice (Maraskovsky et al., 1996) and also increases circulating DCs in humans (Maraskovsky et al., 2000). All DC progenitors, from short-term hematopoietic cells to pre-DCs, are positive for Flk2, the receptor for FL (Schmid et al., 2010). Accordingly, FL deficient mice show a decrease in DC numbers - as well as in hematopoietic progenitors, B cells and NK cells (McKenna et al., 2000).

Murine bone marrow progenitors cultured with FL differentiate into $CD11c^+$ cells (FL-DCs) (Brasel et al., 2000). FL-DCs are heterogeneous and can be separated into 3 subpopulations based on expression of CD11c, B220, CD24 and SIRPα. From the transcription profile and cell-associated antigen presentation assays, it was suggested that $CD11c^+B220^+$ cells correspond to pDCs, $CD11c^+B220^-CD24^{hi}$ correspond to $CD8^+$ splenic DCs and $CD11c^+B220^-SIPR\alpha^{hi}$ correspond to $CD8^-$ splenic DCs (Naik et al., 2005). Although further functional studies are lacking, using FL to differentiate DCs appears to generate cells that strongly resemble conventional steady state DCs.

Dendritic Cell Function

T cells recognize antigen when the T cell receptor (TCR) binds MHC-peptide complexes: $CD4^+$ T cells bind MHCII-peptide complexes, and $CD8^+$ T cells bind MHCI-peptide complexes. TCR binding to cognate MHC-peptide complex initiates a signaling cascade that triggers calcium flux and changes in gene transcription on the T cells. Upon antigen

recognition, T cells up-regulate CD69 expression and enter cell cycle. Several factors modulate T cell activation and cause T cells to differentiate into distinct classes of effector cells: cytokines, density of MHC-peptide complexes; affinity between the TCR and MHC-peptide complexes; and co-stimulatory molecules that stabilize the immunological synapse or deliver additional signals.

In the steady state, DCs capture antigens and present them to T cells, ensuring tolerance by promoting regulatory T cell development or deletion and anergy of self-reactive T cells (Steinman et al., 2003). DCs can promote deletion or anergy of T cells, when presenting antigens in the steady state (Hawiger et al., 2001; Hawiger et al., 2004; Miller et al., 1998). One mechanism proposed is antigen presentation without proper T cell co-stimulation, e.g. ligation of CTLA-4 and PD-1, instead of CD28 (Probst et al., 2005). DCs can also promote the differentiation and survival of regulatory T cells (Darrasse-Jeze et al., 2009; Kretschmer et al., 2005; Yamazaki et al., 2008). Lamina propria DCs induce regulatory T cell conversion by tumor growth factor (TGF)-β and retinoic acid dependent mechanisms (Coombes et al., 2007; Sun et al., 2007).

Importantly, DCs have an innate sensing function, reacting to potential pathogens and changing the context in which they present antigens to T cells in order to initiate adaptative immune responses. Hence DCs regulate both the induction of immunological tolerance and immunity.

DCs express pathogen-recognition receptors such as Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors, retinoic acid inducible gene (RIG)-I-like receptors, as well as lectins (Joffre et al., 2009). Ligation of TLRs, for example, leads to signaling cascades involving the intracellular adaptors MyD88 (myeloid differentiation primary response gene 88) and/or TRIF (TIR domain-containing adapter-inducing interferon-β) that activate transcription factors involved in inflammation (e.g. NFκB, AP-1, IRF3). The concerted action of these transcription factors is responsible for the expression of innate response genes (pro-inflammatory cytokines and chemokines). TLR ligation also increases membrane expression of molecules that optimize T cell activation such as MHC-peptide complexes and co-stimulatory molecules (B7 family members). These phenotypic changes on DCs, from a naïve state to an activated state, are termed maturation. Maturation can also be induced by inflammatory cytokines such as TNFα, IL-1β or IL-6, or by interaction with T cells through CD40-ligation (Caux et al., 1994; Gallucci and Matzinger, 2001). Antigen presentation by mature DCs leads to T cell activation into effector cells (Guermonprez et al., 2002).

Distinct effector $CD4^+$ T cells secrete different cytokines that impact upon host resistance and immunopathology. The class of effector T cell responses is dictated both by the DC subset and by the stimuli that triggered DC maturation (Pulendran et al., 2008). Upon TLR ligation, splenic $CD8^+$ DCs produce IL-12 and express CD70 (Soares et al., 2007), activating T cells to produce IFN-γ (Th1 responses). Splenic $CD8^-$ DCs elicit Th2 polarized T cell differentiation (IL-4, IL-5 and IL-13 production) (Maldonado-Lopez et al., 1999; Maldonado-Lopez et al., 2001; Soares et al., 2007). Nonetheless, splenic $CD8^-$

DCs can also induce Th1 responses, when IL-12 is present (e.g. produced by $CD8^+$ DCs) (Soares et al., 2007) or by expression of Delta 4 Notch-like ligand (Skokos and Nussenzweig, 2007). DCs respond to environmental cues, secreting different cytokines and expressing distinct co-stimulatory molecules that modulate T cell differentiation. For example, epithelial cells can produce thymic stromal lymphopoietin (TSLP) that inhibits IL-12 production and induces OX-40L expression on DCs, favoring Th2 responses (Ito et al., 2005; Soumelis et al., 2002; Taylor et al., 2009). Alternatively, factors secreted by parasites can also directly modulate DCs to induce Th2 responses (Everts et al., 2009; Massacand et al., 2009; Steinfelder et al., 2009).

Dendritic Cells and Lectins

Dendritic cells express several different lectins and lectin-like receptors (carbohydrate-binding proteins) (Dudziak et al., 2007; Robinson et al., 2006). Many of these lectins are members of the Ca^{2+}-dependent C-type lectin family that function in cell adhesion (cell-cell contacts and migration), pathogen recognition and antigen uptake (Cambi and Figdor, 2003). For example, DC-SIGN has been implicated in DC migration, adhesion to T cells, (Geijtenbeek et al., 2000; van Kooyk and Geijtenbeek, 2002), as well as binding to human immunodeficiency virus and other pathogens (Cambi et al., 2003; Engering et al., 2002; Geijtenbeek and van Kooyk, 2003; van Die et al., 2003).

C-type lectins can be classified in groups based on their molecular structure. Type VI lectins are type I transmembrane proteins (intracellular C-terminus) with large extracellular domains containing a membrane distal cysteine-rich domain, followed by a fibronectin type II domain and 8 to 10 C-type lectin like domains. Members of this family include the phospholipase A2 receptor, the (macrophage mannose receptor) MMR and DEC-205. Type II lectins have a single extracellular C-terminal C-type lectin-like domains and include DC-SIGN, Langerin, BDCA-2, Dectin and DCIR (Figdor et al., 2002).

Unlike TLRs, which have a defined and conserved intracellular signaling cascade, lectins differ greatly in their intracellular motifs and no common adaptors have been described. Interestingly, crosslinking of BDCA-2 suppresses TLR-induced type-I interferon production by pDCs (Dzionek et al., 2001). Other lectins can also suppress TLR signaling (Robinson et al., 2006). On the contrary, engagement of Dectin-1 (Brown, 2006; Leibundgut-Landmann et al., 2008) or CLEC9A (Caminschi et al., 2008) triggers signaling cascades that promote DC activation and consequently T-cell immune responses.

Antigen Targeting to Dendritic Cells

Antigen delivery to DCs *in vivo* was pioneered using antibodies that bind to DEC-205, a multilectin endocytic receptor that is highly expressed by $CD8^+$ DCs (Bonifaz et al., 2002; Boscardin et al., 2006; Hawiger et al., 2001; Hawiger et al., 2004; Trumpfheller et al., 2006). Recently, a similar strategy of antigen targeting to the $CD8^-$ DC subset was developed with anti-DCIR2 antibodies (Dudziak et al., 2007; Nussenzweig et al., 1982).

Targeting antigen to DCs increases presentation to T cells by 100-fold (Hawiger et al., 2001). Antigen targeting to DEC-205 or DCIR-2 in the steady state leads to the induction of immunological tolerance, whereas, in the presence of maturation stimuli, such as anti-CD40, it results in immunity (Bonifaz et al., 2002; Dudziak et al., 2007; Hawiger et al., 2001; Steinman et al., 2003).

Antigen delivery to several other molecules expressed by DCs also promotes antigen presentation to T cells. Examples include Dectin-1 (Carter et al., 2006b), Dectin-2 (Carter et al., 2006a), DNGR-1 (Sancho et al., 2008), langerin (Idoyaga et al., 2008) and MHCII (Dickgreber et al., 2009), among others.

DEC-205

The monoclonal anti-DEC-205 antibody (rat IgG2a, NLDC-145) was first described in 1986, recognizing a 145Kd antigen expressed by "non-lymphoid DCs" (Kraal et al., 1986). It was re-named DEC-205 due to its rectified molecular weight of 205 Kd and expression on dendritic and epithelial cells (Jiang et al., 1995; Swiggard et al., 1995).

Anti-DEC-205 (anti-DEC) antibodies are quickly endocytosed in clathrin-coated vesicles and delivered to an intracellular compartment where antigen processing for antigen presentation to T cells ensues (Jiang et al., 1995). When DEC-205 and the MMR are compared, a distinct intracellular distribution is observed. DEC-205 has higher co-localization with LAMP-1 and MHCII positive compartments. In addition, DEC-205 is superior to MMR in mediating antigen presentation to $CD4^+$ T cells. Using a heterologous system, the differences between DEC-205 and the MMR were ascribed to an acidic amino acid cluster (EDE) on the DEC-205 cytoplasmic tail (Mahnke et al., 2000).

In humans, DEC-205 is expressed on monocyte-derived DCs as well as on DCs in the T-cell areas of spleen and lymph nodes (Pack et al., 2008). Antigen targeting to DEC-205 on human monocyte-derived DCs also results in antigen presentation to both $CD8^+$ (Bozzacco et al., 2007) and $CD4^+$ T cells (Gurer et al., 2008).

DCIR-2

The 33D1 antibody (rat IgG2b, anti-DCIR2) was the first DC-specific antibody generated (Nussenzweig et al., 1982), enabling specific depletion of DCs and definitively demonstrating DCs' prominent role as accessory cells for the mixed leukocyte reaction (Steinman et al., 1983) and in T-cell dependent antibody production (Inaba et al., 1983). The combination of 33D1 and anti-DEC-205 staining, among other markers, revealed that there were two different kinds of splenic DCs (Crowley et al., 1989).

Through a combination of gene array and candidate gene approaches, DCIR2 (*Clec4a4*) was identified as the antigen for the 33D1 antibody (Dudziak et al., 2007). DCIR is an abbreviation for dendritic cell inhibitory molecule. The family consists of 4 members: DCIR1, DCIR2, DCIR3, DCIR4 (Flornes *et al., 2004*). Both DCIR1 and DCIR2 contain an inhibitory ITIM motive in the intracellular domain. Most studies involving murine DCIR are referring to DCIR1 (*Clec4a2*) (Bates et al., 1999; Fujikado et al., 2008; Kanazawa et al., 2004), and no further studies with DCIR2 have been reported so far. In humans, by sequence analysis, just one inhibitory C-type lectin gene exists and is denominated ClecSF6 (Bates *et al.,* 1999).

Antigen Uptake

DCs possess several mechanisms to sense and sample the environment. DCs acquire antigens through pinocytosis, phagocytosis or receptor mediated endocytosis.

Pinocytosis (or macropinocytosis) is a constitutive process in DCs and can be induced, e.g. by growth-factors in other cell types. It is initiated by membrane ruffling (actin-rich extensions of the plasma membrane) that collapse and internalize extracellular material. The internalized material (usually fluid) and membrane form large vesicles (greater than 0.2 μm in diameter) that follow a similar intracellular pathway as other endocytic vesicles (Kerr and Teasdale, 2009).

Phagocytosis is also an actin-dependent process, but it involves local membrane activation for the uptake of large (greater than 0.5 μm) particulate matter. It is an evolutionary conserved immune-related process, specific to a few cell types such as macrophages, neutrophils and DCs. Particle opsonization by antibodies or complement fixation enhance phagocytosis (Greenberg and Grinstein, 2002). DCs, particularly the $CD8^+$ subset, are very efficient in phagocytosis of apoptotic cells (Iyoda et al., 2002).

In addition, DCs display complement, scavenger and Fc-receptors, as well as C-type lectins that can bind pathogens and other antigens. Receptor-mediated endocytosis typically occurs from coated pits, by recruitment of dynamin and adaptor proteins to form clathrin-coated vesicles. Caveolin containing invaginations or clathrin/caveolin independent internalization may also occur is some specific situations, but the contribution of these processes to antigen presentation remains unexplored (Conner and Schmid, 2003; Mellman, 1996).

DC activation induces an initial increase in pinocytosis (West et al., 2004), followed by a gradual decrease over 1-2 days (Sallusto et al., 1995). Inhibition of antigen capture by mature DCs is attributed to inactivation of Cdc42, a Rho GTPase family member that promotes actin polymerization (Garrett et al., 2000). Receptor-mediated endocytosis is not affected by DC maturation (Garrett et al., 2000; Platt et al., 2010).

Upon internalization, antigens are sorted and delivered into different compartments depending on adaptor proteins recruited during the internalization process (Lakadamyali et al., 2006). Early endosomes are mildly acidic (pH of ≈6.0) and rely on the small GTPase Rab5, early endosome antigen 1 (EEA1) and phosphoinositide 3-kinase for their formation and function. As a consequence of the low pH, some ligands dissociate from their receptors and mix with internalized solutes. Membranes pinch off from early endosomes and fuse back to the plasma membrane, or go to Rab4 and Rab11 positive endosomes (recycling endosomes). Thus membrane proteins that do not have specific motifs for lysosomal delivery are usually diverted back to the plasma membrane. Proteins containing tyrosine-based and dileucine-based amino acid motifs, or ubiquitylation of cytoplasmic domains, are directed to a degradative pathway; they go to acidic late endosomes/multivesicular bodies (Rab7 positive) and lysosomes (pH<5, where catabolism occurs) (Bonifacino, 2003; Maxfield and McGraw, 2004; Saftig and Klumperman, 2009). Alternatively, some proteins can engage in a retrograde transport pathway, from either early or late endosomes, that results in sorting into the Golgi complex. For example, in order to gain access to the Golgi and the ER, toxins exploit retrograde transport mechanisms (Johannes and Popoff, 2008).

Endosomes are a very flexible and complex network, where several intermediate stages exist: vesicles exchange contents gradually and "mature" over time. Therefore the division into early and late endosome is only a simplification of the process.

Antigen Processing and Presentation

Constitutive MHCII expression characterizes professional antigen presenting cells: DCs, B cells, macrophages and thymic epithelial cells. After internalization, exogenous antigens reach endosomal compartments where they are digested into peptides that can associate with MHCII molecules.

MHCII molecules are guided to endosomes by the invariant chain (Ii). In the endoplasmic reticulum (ER), MHCII molecules (heterodimers of α-chain and β-chain) associate with Ii trimers, forming nonamers $(\alpha\beta Ii)_3$. This association blocks the peptide-binding groove on MHCII molecules and guides the complex through the Golgi apparatus into endosomes. Alternatively, Ii-MHC complexes go transiently to the plasma membrane and are internalized back into endosomes. The acidic environment of endosomes triggers Ii degradation by cathepsins, leaving only a small portion that blocks the peptide-binding groove (CLIP, Ii derived MHC class II-associated invariant chain peptide). CLIP is displaced when MHCII molecules interact with catalyst HLA-DM/H2-M (in humans/mice), allowing binding of antigenic peptides present in endosomal compartments. MHCII-peptide complexes then travel to the plasma membrane to mediate antigen presentation (Jensen, 2007; Landsverk et al., 2009; Trombetta and Mellman, 2005).

Intracellular antigens can also be delivered to lysosomes and have access to MHCII presentation in a process denominated autophagy (Munz, 2010; Paludan et al., 2005). Autophagy as means to promote MHCII presentation of intracellular antigens is an

important physiological process in thymic epithelium with great impact in $CD4^+$ T cell tolerance (Nedjic et al., 2008), and it also occurs constitutively in a myriad of other cell types, including DCs (Schmid et al., 2007).

Unlike MHCII, all nucleated cells express MHCI molecules. Classical MHCI presentation occurs after degradation of ubiquitylated intracellular proteins by the proteasome, generated for example from the catabolism of endogenous proteins. Peptides generated by the proteasome are degraded by cytoplasmic peptidases or shuttled into the ER by the TAP complex (transporter associated with antigen processing). In the ER, calnexin assists MHCI heavy chain assembly with the invariant β2-microglobulin, and peptides can be loaded onto nascent MHCI molecules with the assistance of chaperones (tapasin, calreticulin). ER amino peptidases (ERAP) are responsible for the final trimming of peptides for MHCI loading. MHCI-peptide complexes leave the ER and travel through the Golgi complex to the plasma membrane (Jensen, 2007).

Extracellular antigens can also be loaded onto MHC class I molecules in a process called cross-presentation. The cellular mechanisms responsible for cross-presentation are not resolved and at least 3 different intracellular routes have been proposed: (1) cytosolic, TAP-dependent pathway in which antigens (or peptides) escape from endosomes, into the cytoplasm, where they are degraded by the proteasome or directly shuttled into the ER by TAP; (2) retrograde-like pathway in which ER components fuse with endosomes during the internalization process; (3) vacuolar, TAP-independent pathway in which endosomes

containing exogenous proteins and peptides can fuse with endosomes containing recycling MHCI molecules (Guermonprez et al., 2002; Jensen, 2007).

The current view is that MHCII presentation of exogenous antigens is more robust than cross-presentation, since it can occur in either early and recycling endosomes (with low proteolytic activity/neutral-low pH), where newly generated peptides associate with recycling MHCII molecules, or occur in late endosomes (high proteolytic activity/low pH), where antigenic peptides displace CLIP, and are loaded into nascent MHCII molecules. In opposition to MHCII presentation, cross-presentation is favored when the antigen is retained in early and recycling endosomes (Belizaire and Unanue, 2009; Savina et al., 2006).

The mechanism of antigen uptake determines the recruitment of adaptor proteins and thus the nature of the intracellular compartment reached. Distinct intracellular compartments have different pH and proteases, affecting the generation of peptides. Also, as described above, MHC molecules traffic between different compartments, and peptide loading occurs in a few favorable environments. Thus the intracellular compartments reached by the antigens profoundly impact antigen processing and presentation. Ovalbumin (OVA) internalization through the MMR in GM-DCs, results in antigen retainment in early endosomes and exclusive cross-presentation. OVA internalization, through scavenger receptors in bone marrow derived macrophages, results only in late endosomal delivery and MHCII presentation (Burgdorf et al., 2007). Internalized soluble OVA can escape to the cytosol where it undergoes proteasomal processing and peptides reach MHCI

molecules by a TAP-dependent pathway. In contrast, internalized soluble hen egg lysozyme (HEL) does not reach the cytoplasm; conversely, it travels to late endosomes compartments, and no HEL cross-presentation can be detected. Nonetheless, HEL entrapped in liposomes, with early endosomal release, is processed and loaded into MHCI within early endosomes (vacuolar pathway) (Amigorena and Savina, 2010; Belizaire and Unanue, 2009)

Cell intrinsic factors (expression of proteases, ATP pumps and even route of MHC molecules) can also impact antigen presentation. When compared to macrophages, GM-DCs have low levels of lysosomal proteases (Delamarre et al., 2005; Lennon-Dumenil et al., 2002), which correlate with higher formation of MHC-peptide complexes (Delamarre et al., 2006). Immature GM-DCs control protein degradation by limiting V-ATPase assembly, thus preventing endosomal acidification (Trombetta et al., 2003). In addition the recruitment of the NADPH oxidase NOX2 into endosomes prevents acidification, and NOX2 deficient DCs are less efficient at cross-presentation (Savina et al., 2006).

$CD8^+$ DCs are superior at cross-presenting antigens after ingestion of dead cells or during viral infection (Belz et al., 2005; Hildner et al., 2008; Jirmo et al., 2009), and cell-intrinsic mechanisms have been proposed by various groups (Amigorena and Savina, 2010). NOX2 is specifically recruited in $CD8^+$ DCs phagosomes, but not in $CD8^-$ DCs, and NOX2 recruitment is responsible to avoid phagosomal acidification and therefore favors antigen cross-presentation (Savina et al., 2009). Cytochrome C administration to mice is toxic to $CD8^+$ DCs, but not $CD8^-$ DCs, suggesting that $CD8^+$ DCs have unique

mechanisms to transport exogenous antigens into the cytosol (Lin et al., 2008). However definitive data showing the mechanism responsible for $CD8^+$ DC superiority at cross-presentation is still lacking.

Most studies on antigen presentation by DCs were performed with GM-DCs. Although extremely valuable, they are restricted to one particular cell type. In addition, GM-DCs are closer to monocytes than DCs in both mRNA expression profile (Robbins et al., 2008) and phenotype (Xu et al., 2007). Several subsets of DCs can be identified *in vivo*, and their relative contribution to antigen presentation is a matter of intense investigation. Here I compare MHCI and MHCII antigen presentation by resting or activated spleen DCs ($CD8^+$ and $CD8^-$ subsets) after *in vivo* or *in vitro* antigen delivery. I initiated this study by delivering antigens using recombinant antibodies to DC receptors. I also compared receptor-mediated endocytosis to pinocytosis and phagocytosis. To complement my work, I analyzed the antigen presentation efficiency of activated monocytes, GM-DCs and FL-DCs, as well as B cell blasts.

Chapter 2

Materials and Methods

Mice

C57BL/6, B10.BR, C3H/HeJ and Balb/c were purchased from Jackson Laboratory. Unless stated otherwise, C57BL/6 mice were used. TCR transgenic OTII and OTI mice, specific for OVA peptide in the context of IA^b and $H2K^b$, respectively, were bred to C57BL/6 CD45.1 mice. Transgenic TCR specific for $H2K^d$-SYVPSAEQI (peptide from *Plasmodium yoelii* circumporozoite protein)(Sano et al., 2001) and CD11c-hDEC (Dudziak et al., 2007) mice; B1-8^{hi} Immunoglobulin heavy chain knock-in (Shih et al., 2002) mice; DEC-205 deficient (Guo et al., 2000) and GM-CSF receptor-β deficient (Robb et al., 1995) mice were maintained and bred at the Rockefeller University. 6-10-week-old mice were used in experiments. All experiments were performed in accordance with NIH guidelines and approved by The Rockefeller University Animal Care and Use Committee.

Production of chimeric antibodies and other antigens

The following chimeric antibodies were produced: anti-mouse-DEC-OVA, anti-DCIR2-OVA, anti-human-DEC-OVA, Iso-OVA, anti-mouse-DEC-HEL, anti-DCIR2-HEL, anti-human-DEC-HEL, Iso-HEL, anti-mouse-DEC-CSpep (peptide SYVPSAEQI from *Plasmodium yoelii* circumporozoite protein), anti-DCIR2-CSpep, Iso-CSpep, anti-mouse-DEC-CSf (circumsporozoite protein from *Plasmodium falciparum*) and anti-DCIR2-CSf.

Chimeric antibodies were expressed by transient transfection of 293T cells (ATCC) and purified with Protein G (GE Healthcare) as described (Hawiger et al., 2001; Tiller et al., 2008).

OVA (Grade V, Sigma) was decontaminated from LPS by multiple rounds of extraction with triton-X 114 (Sigma) (Aida and Pabst, 1990) and dialysed extensively. NP (4-hydroxy-3-nitrophenil, Biosearch) and Biotin (Invitrogen) were conjugated to OVA according to manufacturers' instructions. All antigens were run in SDS-PAGE, tested for LPS contamination (Fisher-Cambrex) and decontaminated by triton-X 114 extraction when necessary (above 0.005 EU/ μg).

OVA was adsorbed to 2 μM red beads (Polysciences) according to the manufacturer's protocol. Briefly, 0.5 ml of 2.5% suspension of beads was resuspended in 0.1 M borate buffer pH 8.5 and incubated overnight with 500 μg of OVA at 4°C. Alternatively, to obtain 25% OVA-beads, beads were incubated overnight with 125 μg of OVA and 375 μg of keyhole limpet hemocyanin (KLH, Sigma). Unbound protein was washed away extensively, and OVA-adsorbed beads were resuspended in 1 ml PBS. Coupling was confirmed by flow-cytometry with anti-OVA antibodies (rabbit, Cappel) followed by anti-rabbit-Cy5 (mouse, Jackson Immunoresearch).

Antibodies and flow cytometry analysis

The following antibodies were purchased from either BD or eBioscience: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b (M1/70), CD11c (N418, HL3), CD16 and

CD32/Fc block (2.4G2), CD19 (MB19-1 or eBio1D3), CD24/HSA (M1/69), CD45R/B220 (RA3-6B2), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD86 (GL1), CD115/CSF-1R (AFS98), CD135/Flk-2/Flt3 (A2F10), CD172α/SIRPα (P84), mouseCD205/mDEC-205 (205yefta), humanCD205 /hDEC-205 (Mg38), DCIR2 (33D1), Fas/CD95 (Jo2), GL7, H2Kb (AF6-88.5), I-E^k (14-4-4S), Ly6C (AL-21 or HK1.4), Ly6G (1A8), MHCII (AF6-120.1 or M5/114.15.2), NK1.1 (PK136), Ter-119, Thy1.1 (OX-7), Vα2 (B20.1) and anti-mouse IgG1 (A85-1). Streptavidin-PE and –PE-Cy5.5 were from eBioscience. Streptavidin APC was from BD. Streptavidin Pacific Blue and DAPI were from Invitrogen. Anti-OVA was from Cappel. Anti-mouse IgG, anti-mouse IgG1, anti-rat and anti-rabbit were from Jackson Immunoresearch. Anti-rat-PE was from Santa Cruz. Antibodies to DEC-205 (NLDC-145), DCIR2 (331D1, DC), IA^k-HEL (Aw1.18.14) (Dadaglio et al., 1997) and *Plasmodium falciparum* circumporozoite protein (2A10) (Hollingdale et al., 1984), were purified from hybridomas and labeled with biotin, Alexa Fluor 488 or Alexa Fluor 647 (Molecular Probes). Isotype controls rat IgG2a and rat IgG2b biotin, Alexa Fluor 488 and Alexa Fluor 647 were from Molecular Probes.

Cell suspensions were incubated in PBS 2% FCS (fetal calf serum, Gibco) with purified Fc block and surface staining was performed with indicated antibodies for 20-60 minutes on ice. Data was acquired on Calibur or LSR-II (BD). Analysis was performed using Diva (BD) or FlowJo (TreeStar).

Immunofluorescence

Splenic section were fixed and stained as described previously (Lindquist et al., 2004). Briefly, spleens from C57BL/6 mice were fixed in PBS with 4% paraformaldehyde and 10% sucrose; cryoprotected in 30% sucrose; embedded in optimum cutting temperature compound and frozen at -80°C. Frozen tissues were sectioned (10-20 μm thickness) on a microtome and fixed in acetone. All incubations were done in a humidified chamber. Sections were blocked in 5% BSA in HBSS. The primary antibodies were IgD-Alexa Fluor 568 (eBioscience/in house labeling with Invitrogen kit), CD3-FITC (BD) and chimeric anti-DCIR2-CSf, anti-DEC-CSf or Iso-CSf. Secondary antibody was anti-CSfalciparum-Alexa Fluor 647 (in house production). Sections were mounted in Fluoromount-G (Southern Biotech). Confocal images were acquired on a Zeiss LSM 510 system equipped with 488, 543 and 633 nm excitation lines at The Rockefeller University Bio-Imaging Facility. Images were obtained with a 20x Plan Apochromat (NA 0.75) objective. Images were processed in Adobe Photoshop.

Detection of chimeric antibodies by flow cytometry

DCIR2 binding is calcium dependent, so EDTA was omitted in all steps. Anti-mouseDEC and anti-DCIR2 chimeric antibodies have a mouse IgG1 constant region. For extracellular detection, splenocytes were stained 15 min on ice with anti-mouse IgG1 FITC (A85-1, BD) or anti-mouse IgG1 Cy5 (goat polyclonal, Jackson Immunoresearch) in PBS 1% BSA 0.05% azide. Cells were washed and fixed (Cytofix/Cytoperm, BD). For total chimeric antibody detection, splenocytes were stained, after fixation, with anti-mouse IgG1 FITC or Cy5 in Perm/Wash (BD). For intracellular chimeric antibody

detection, extracellular signal was blocked with saturating amounts of unlabeled anti-mouse IgG1 and after washing, cells were fixed and stained with anti-mouse IgG1 FITC or Cy5 in Perm/Wash. Cells were subsequently stained with antibodies to CD11c, CD8α and CD4 to identify DC populations.

In some experiments, rat monoclonal anti-DEC (NLDC) or anti-DCIR2 (33D1) were used. Antibody detection was with anti-rat Cy5 (mouse $F(ab')_2$, Jackson Immunoresearch) with similar protocol as described above.

Detection of chimeric antibodies by Western blot

Splenic DCs were enriched with anti-CD11c beads (Miltenyi) according to manufacturer's instructions. Enriched DCs were incubated on ice with chimeric anti-DEC-CSf or anti-DCIR2-CSf at 5 μg/ml, and excess antibodies were removed by washing. DCs were either kept on ice or incubated for 10-180 minutes at 37°C in complete RPMI media (RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM L-Glutamine, Antibiotic/AntiMycotic, 1 mM Sodium Pyruvate and 53 μM 2-ME, all from Gibco). To remove extracellular antibodies, DCs were washed with RPMI buffered to pH 3.6 with citric acid. $2x10^6$ DCs were lysed in 1% NP-40, 150 mM NaCl, 10mM TrisHCl pH8 buffer containing EDTA-free protease-inhibitor cocktail (Roche), incubated on ice for 30 min, and debris spun out at 14,000 rpm, 4°C. Samples were separated on 4-12% acrylamide Bis/Tris/SDS gels (Invitrogen), transferred to PVDF membranes (Millipore) and blotted with biotinilated anti-CS falciparum antibodies (in house production) followed by steptavidin-HRP (Jackson Immunoresearch). Membranes were

blotted with anti-β-actin (Sigma) followed by anti-mouse-HRP (Jackson Immunoresearch) as loading control. Western blots were developed using enhanced chemiluminescent substrate (ECL, Pierce).

In vivo antigen administration

Mice were injected intraperitoneally (i.p.) or intravenously (i.v.) with chimeric antibodies as indicated in each experiment. In some experiments 50 μg agonistic anti-CD40 monoclonal antibody (MSKCC, Rockefeller Monoclonal Antibody Core Facility), or 30 μg LPS (*E.coli* Serotype 0111:B4, Sigma) were co-injected with chimeric antibodies. To induce B cell germinal centers, mice were immunized with 100 μg KLH (Sigma) in alum (Thermo Scientific) 9 days before analysis.

Adoptive transfer and T cell proliferation responses

$CD8^+$ and $CD4^+$ OVA-specific T cells were isolated from OTI and OTII mice respectively. $CD8^+$ *Plasmodium yoelii* CS-specific T cells were isolated from mice with transgenic TCR specific for $H2K^d$-SYVPSAEQI. $CD8^+$ and $CD4^+$ T cells were enriched with a $CD8^+$ T cell isolation kit or a $CD4^+$ T cells isolation kit, according to the manufacturer's instructions (Miltenyi) with the addition of anti-CD11c-biotin to the antibody cocktail. Enriched T cells were labeled with 3 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE, Molecular Probes) in PBS 0.1% BSA for 10 min at 37°C. The reaction was quenched with FCS and cells were washed 2-3 times. Enriched T cells (1 to 4 x 10^6) were injected intravenously into recipient mice. 24 hours later antigen was injected intraperitoneally. In experiments addressing antigen persistence, chimeric

antibodies containing antigen were injected 10, 7, 5, 3 or 1 day before T cell transfer. Proliferation of transferred T cells was analyzed after 3 days.

Production of retroviruses

DCIR2 was cloned into pMX-PIE vector carrying an IRES-GFP and puromycin resistance gene. DEC-205 was cloned into a modified pMX-PIE vector containing puromycin resistance gene, but no IRES-GFP. Deletions on the cytoplasmic tail of DEC-205 were introduced by overlapping PCR and alanine substitutions were introduced by site-directed mutagenesis PCR. All plasmids were verified by sequencing. BOSC 23 cells were cotransfected with pMX-PIE vector and pCL-ECO plasmids and supernatants containing virus were harvested after 48 and 72 hours. Cells were spin-infected with retrovirus containing filtered supernatants mixed with 10 μg/ml polybrene (Sigma) and 20 mM HEPES.

Antigen presentation assay with transduced GM-DCs

Bone marrow cells were obtained by flushing femurs and tibiae with RPMI supplemented with 5% FCS. Red blood cells were removed by ACK lysis (Gibco) and washed with complete RPMI.

For GM-DCs differentiation (Inaba et al., 1992a), bone marrow cells were plated at 1.5 x10^6/ml in complete RPMI with 3% vol/vol supernatant of J558L cells transduced with murine GM-CSF (provided by A. Lanzavecchia). Cells were cultured at 37°C and 5% CO_2. Media was changed every 2 days, removing loosely adherent and dead cells. GM-

DCs were infected with retrovirus containing supernatants on day 2, 3 and 4. On day 6 transduced GM-DCs were assayed for expression of DCIR2, DEC-205 and GFP, and $CD11c^+GFP^+$ and $CD11c^+GFP^-$ cells were purified by cell sorting. Purified cells were incubated for 16 hours with 1 μg/ml anti-DCIR2-OVA, anti-DEC-OVA or Iso-OVA, and 100 ng/ml LPS for further 12 hours. Cells were then washed and co-cultured with OT-II T cells, and T cell proliferation was determined by [^{3}H]-thymidine incorporation 48 hours later.

Antigen presentation and internalization assays with transduced B cells

Naïve B cells were isolated from DEC-205 deficient mice. Single cell suspensions from spleen were incubated with anti-CD43 beads (Miltenyi) and enriched in LS columns to obtain $CD43^-$ naïve B cells. Enriched B cells were plated at 0.7 $x10^6$ cells/ml in complete RPMI supplemented with 25 μg/ml LPS (Sigma) and 5 ng/ml IL-4 (Sigma) and cultured 37°C and 5% CO_2. B cells were infected with retrovirus-containing supernatants on day 1 and 2. Transduced B cells were selected with puromycin from day 3 to 5, and live B cell blasts separated with Ficoll-Paque Plus (GE Healthcare) gradient.

To measure cell associated OVA, 60,000 B cells were incubated with anti-DEC-OVA (10, 30, 100, 300, 1,000, 3000, 10,000 ng/ml) for 20 min on ice. Excess unbound antibody was removed by washing, and B cells were fixed and stained with rabbit anti-OVA (Cappel) followed by anti-rabbit Cy5 (mouse, Jackson Immunoresearch). OVA content was quantified by flow cytometry based on mean fluorescence intensity. For

antigen presentation assessment 30,000 anti-DEC-OVA pulsed B cells were incubated with CFSE labeled OTI or OTII T cells for 3-4 days as described bellow.

To measure DEC internalization, transduced B cells were incubated with anti-DEC-Alexa Fluor 647 (NLDC, rat monoclonal, in house labelling) on ice for 20 min. Excess antibody was removed, and cells were either kept on ice or incubated for 90 min at 37°C. Extracellular DEC-205 was detected with anti-rat PE (goat $F(ab')_2$, Santa Cruz). Internalization was calculated for DEC (Alexa Fluor 647)$^+$ B cells according to the following formula: 100- [MFI anti-rat PE (90 min at 37°C) x 100] / MFI anti-rat PE (ice).

Isolation and sorting strategy for DCs and activated monocytes

Dendritic cells were isolated from mice that were injected with 1-2 10^6 B16-FL melanoma cells 9-12 days before euthanasia (Mach et al., 2000). All experiments were also performed, at least once, with dendritic cells isolated from naïve mice (no FL administration), with identical results. For *in vivo* targeting experiments, mice were injected intraperitoneally with 10 μg of fusion antibodies 8-12 hours before euthanasia.

Activated DCs and monocytes were isolated from mice that were primed and boosted with methylated BSA (mBSA) in complete Freund's adjuvant (CFA, Difco) and then injected intraperitoneally 24 hours before analysis with 100 μg mBSA (Sigma) as described (Cook et al., 2004; Naik et al., 2006). Tip-DCs were isolated from mice intravenously injected with 3,000 *Listeria monocytogenes* (gift from E. Pamer), 45 hours before euthanasia (Busch et al., 2001). *L. monocytogenes* was grown in brain hearth

infusion broth until early log-phase, monitored by light absorbance (A_{600}) and ascertained by colony counting after plating into agar plates.

Spleens were removed, injected with 0.4 U/ml collagenase D type II (Roche) in HBSS (Gibco) with 2% FCS and cut into small fragments, before 30 min digestion at 37°C. Digestion was stopped, with 5 mM EDTA (Gibco) for 5 min, before collection of cell suspensions. Red blood cells were removed by ACK lysis (Gibco). All subsequent steps were performed in PBS 2% FCS and during incubations, cells were kept on ice. Non-specific binding was blocked with purfied Fc block and DCs were enriched with anti-CD11c beads (Miltenyi) according to manufacturer's instructions. Enriched naïve $CD11c^+$ cells were stained and sorted as follow: $CD8^+$ DCs ($B220^-$, $NK1.1^-$, $CD11c^{hi}$, $CD8^+$) and $CD8^-$ DCs ($B220^-$, $NK1.1^-$, $CD11c^{hi}$, $CD8^-$, $CD4^+$). Enriched $CD11c^+$ cells from immunized mice were stained and sorted as follow: iMono ($CD8^-$, $CD11b^{hi}$, $CD11c^{int}$ and $Ly6C^+$), $iCD8^-$ DCs ($CD8^-$, $Ly6C^-$, $CD11b^{int}$ and $CD11c^{hi}$) and $iCD8^+$ DCs ($CD8^+$, $CD11c^{hi}$ and $Ly6C^-$). In mice infected with *L. monocytogenes,* Tip-DCs were enriched with CD11c and CD11b beads and sorted as $CD8^-$, $CD11b^{hi}$, $CD11c^{low/int}$ and $Ly6C^+$. Cells were also stained with B220, NK1.1, CD3, Ter119, Ly6G to gate out non-DCs/non-monocytes. In addition, in experiments with CD11c-hDEC mice, cells were stained with humanCD205 to sort positive cells. Sorted populations were collected in RPMI with 10% FCS, and live cells were typically >95% pure.

Culture and sorting strategy for DCs cultured from bone marrow progenitors

GM-DCs were cultured as described above and collected at day 7. Non-specific binding was blocked with Fc block and cells were stained with CD11c. $CD11c^{hi}$ cells were sorted.

For FL-DC differentiation (Brasel et al., 2000), bone marrow cells were plated at 1.5 $x10^6$/ml in complete RPMI with 100 ng/ml murine FL. FL was produced by anti-FLAG purification of supernatant from Chinese hamster ovary (CHO) cells expressing recombinant murine FL-FLAG (kindly provided by C.G. Park, The Rockefeller University). Cells were cultured at 37°C and 5% CO_2 for 10 days. FL-DCs were collected, non-specific binding was blocked with purified Fc block and cells were stained and sorted as follow: FL-$CD8^-$DCs ($B220^-$, $CD11c^{hi}$, $SIRP\alpha^{hi}$ and $CD24^{lo}$) and FL-$CD8^+$DCs ($B220^-$, $CD11c^{hi}$, $SIRP\alpha^{lo}$ and $CD24^{hi}$) (Naik et al., 2005).

In vitro activation of DCs was by addition of 1µg/ml LPS (Sigma) for the last 18 hours of culture. In experiments with CD11c-hDEC mice, cells were also stained with humanCD205 to sort positive cells. Sorted populations were collected in RPMI 10% FCS and live cells were typically >95% pure.

B cell culture

Naïve B cells were isolated from WT C57BL/6 or $B1\text{-}8^{hi}$ mice, as indicated. For WT single cell suspensions from spleen were incubated with anti-CD43 beads (Miltenyi) and enriched in LS columns to obtain $CD43^-$ naïve B cells. For $B1\text{-}8^{hi}$ mice, single cell suspensions from spleen and skin-draining lymph nodes were incubated with anti-Igκ-PE (187.1, BD) followed by anti-PE beads (Miltenyi) and anti-CD43 beads (Schwickert et

al., 2007). Cells were enriched in LS columns (Miltenyi) to obtain $CD43^{-}Ig\kappa^{-}$ naïve λ^{+} B cells (NP-specific). Enriched B cells were plated at 0.7 $x10^{6}$ cells/ml in complete RPMI supplemented with 25 μg/ml LPS (Sigma) and 5 ng/ml IL-4 (Sigma) and cultured 37°C and 5% CO_2 for 2-5 days. Alternatively, where indicated, B cells were cultured with 5 μg/ml anti-CD40 (IC10 clone, purified from hybridoma).

***In vitro* antigen delivery**

Sorted APCs were counted, and 15,000 live cells cells/well were plated in complete RPMI in 96-well round bottom plates. For B cell blasts, 30,000 cells/well were plated. Antigen was added at the indicated concentrations. For receptor-mediated endocytosis (DEC-205 targeting and BCR targeting), all media was kept ice cold and antigen was pulsed for 20 min on ice. For pinocytosis, warm media with antigen (OVA-biotin) was added and plates were incubated at 37°C with 5% CO_2 for 2 hours. As controls in all experiments, APCs were also incubated with peptides for 20 min on ice: EQLESIINFEKLTEW peptide for OTI co-culture, and LSQAVHAAHAEINEAGR peptide for OTII co-culture, synthesized by the Proteomics Resource Center, The Rockefeller University. Cells were washed 3 times with complete RPMI to remove excess antigen or peptide. All incubations were done in duplicate wells.

***In vitro* phagocytosis**

Enriched cells from spleen of naïve, immunized or *Listeria* infected mice, or GM-DCs were incubated with OVA-adsorbed fluorescent red beads in complete RPMI for 30 min at 37°C and 5% CO_2. Cells were washed 3 times with cold PBS (900 rcf, 5 min), and

stained as described above. Specific populations containing no beads, 1 OVA-bead or more than 2 OVA-beads were sorted and co-cultured with CFSE labeled OVA-specific T cells.

***In vitro* antigen presentation assay**

$CD8^+$ and $CD4^+$ OVA-specific T cells were isolated from OTI and OTII mice respectively, and labeled with 1 μM CFSE, as described above.

100,000 to 125,000 T cells were added to each well containing APCs in "U" bottom, 96-well plates. Activation and division of OVA-specific T cells was determined by flow cytometry after culture at 37°C and 5% CO_2 for 60-65 hours (OTI cells) or 80-85 hours (OTII cells). For analysis, cells were stained with Vα2 PE, CD69 APC, CD8 or CD4 PerCP and 0.5 μg/ml DAPI before acquisition in a LSR II (BD).

Quantification of antigen capture

APCs were resuspended in Cytofix/Cytoperm (BD) for 15 min at room temperature and washed in Perm/Wash (BD). Anti-DEC-OVA and NP-OVA captured by receptor-mediated endocytosis were detected with rabbit polyclonal anti-OVA (Cappel) followed by anti-Rabbit-HRP (donkey $F(ab')_2$, Jackson Immunoresearch) in PBS 1% BSA 0.1% saponin. OVA-biotin was detected with streptavidin-HRP (Jackson Immunoresearch). HRP content was assessed by a fluorometric assay with amplex red (Invitrogen). Plates were excited at 530 nm and emission was collected at 590 nm on Cytofluor II (Perseptive Biosystems).

Image Stream 100 analysis

$CD8^+$ and $CD8^-$ DCs from naïve mice, or activated monocytes (iMono) from immunized mice, or GM-DCs that had captured a single OVA-bead were sorted as described. After sorting, cells were stained on ice in PBS 1% BSA 0.05% azide with CD11c-, CD11b-, CD8- and MHCII-biotin, followed by streptavidin Pacific blue and analyzed on Image Stream 100 (Amnis). In focus, single cells were gated, and the Pacific blue membrane staining was used to create a mask to enable analysis of internalization according to manufacturer's instructions (Amnis). Histograms were generated showing the distribution of internalization scores where positive scores indicate internalization.

Data analysis

Graphs were compiled on Prism software (GraphPad Software, Inc). Antigen pulse curves representing antigen uptake or T cell proliferation were adjusted to exponential one-phase association curves.

Chapter 3

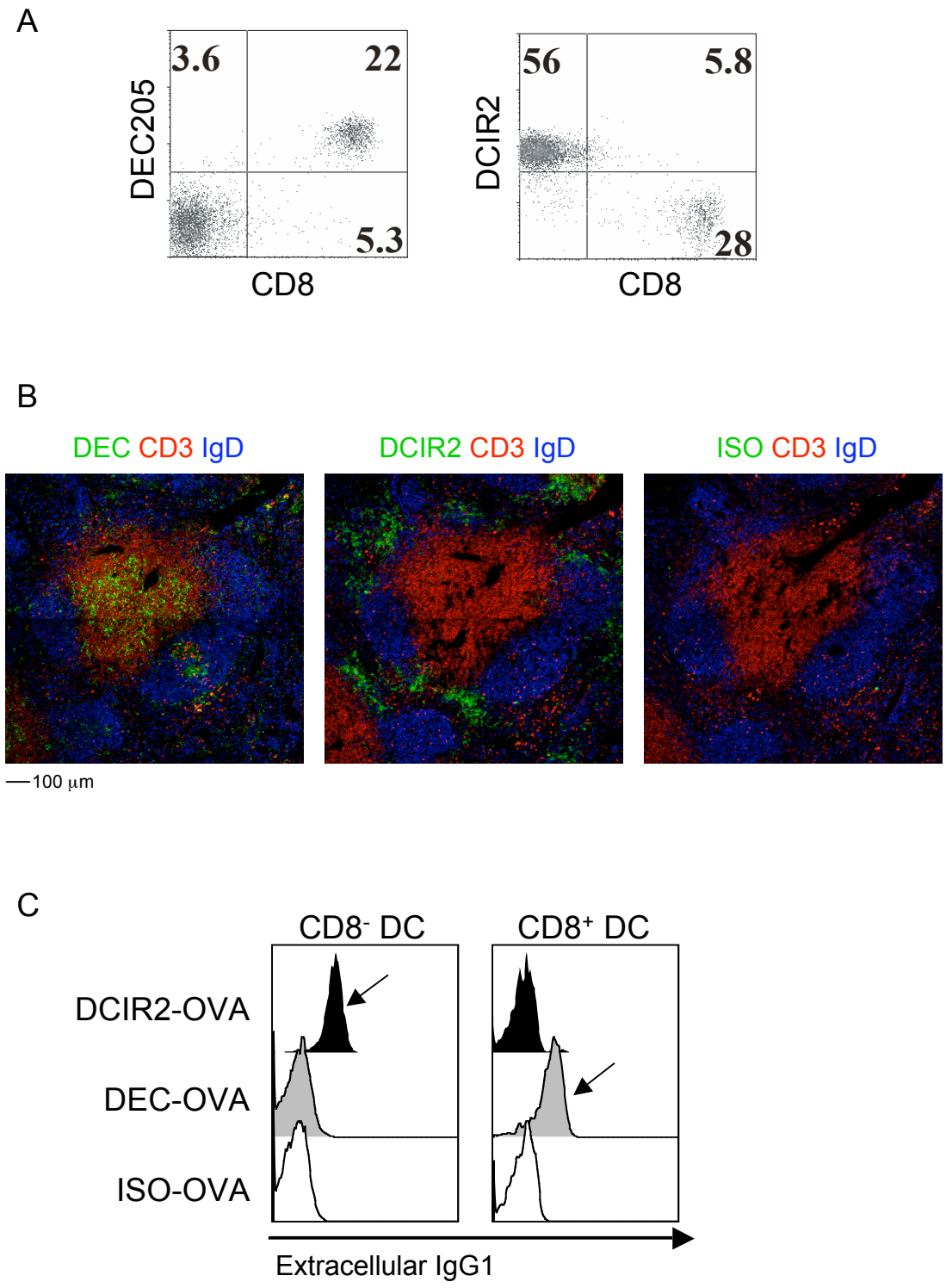
Results Part I

Dendritic cells orchestrate T-cell responses, yet the role of DC subsets in establishing different types of T cell immunity is just beginning to be understood. In murine spleen, two major types of DCs can be identified: $CD8^+DEC\text{-}205^+$ DCs and $CD8^-DCIR2^+$ DCs (Fig. 1A). These DC subsets reside in different anatomic locations: $CD8^+DEC\text{-}205^+$ DCs are prevalent in the T-cell zone, whereas $CD8^-DCIR2^+$ DCs are in the red pulp and marginal zone (Fig. 1B).

When isolated and cultured, DCs can undergo maturation, thus the role of different DC subsets would be best studied *in vivo*. This was facilitated by techniques to target DCs *in vivo,* using chimeric anti-DEC antibodies to deliver antigens to $CD8^+$ DCs (Hawiger et al., 2001). Diana Dudziak identified the DCIR2 lectin as the antigen for the 33D1 antibody, prompting the cloning of the 33D1 antibody to produce chimeric antibodies for antigen delivery to $CD8^-DCIR2^+$ DCs (Dudziak et al., 2007). When anti-DCIR2-OVA antibodies were injected into mice, specific antigen delivery to $CD8^-$ DCs was achieved (Fig. 1C). Anti-DEC-OVA antibodies ensured delivery to $CD8^+$ DCs, and Iso-OVA antibodies did not deliver antigens to DCs (Fig. 1C). Both anti-DEC and anti-DCIR2 antibodies were internalized (Dudziak et al., 2007), albeit with different efficiencies (to be discussed later).

Figure 1. Expression profile and *in vivo* antigen targeting to DEC-205 and DCIR2 A, Dot plots show expression of DEC-205 and CD8α (left) and DCIR2 and CD8α (right) on $CD11c^{hi}$ splenocytes analyzed by flow cytometry. B, panels show immunofluorescence on spleen sections for DEC-205 (left), DCIR2 (center) and Isotype control (left) in green, and CD3 in red (T-cell zone) and IgD in blue (B-cell follicles). C, Histograms show extracellular detection of anti-DCIR2 and anti-DEC antibodies on $CD8^-$ and $CD8^+$ DCs 30 min after intravenous injection of 10 μg of anti-DCIR2-OVA, anti-DEC-OVA, or control Iso-OVA antibodies, visualized with anti-mouse IgG1-FITC. Data is representative of at least 2 independent experiments.

Figure 1


A
3.6
22
5.3
DEC205
CD8
56
5.8
28
DCIR2
CD8
B
DEC CD3 IgD
DCIR2 CD3 IgD
ISO CD3 IgD
100 μm
C
CD8- DC
CD8+ DC
DCIR2-OVA
DEC-OVA
ISO-OVA
Extracellular IgG1

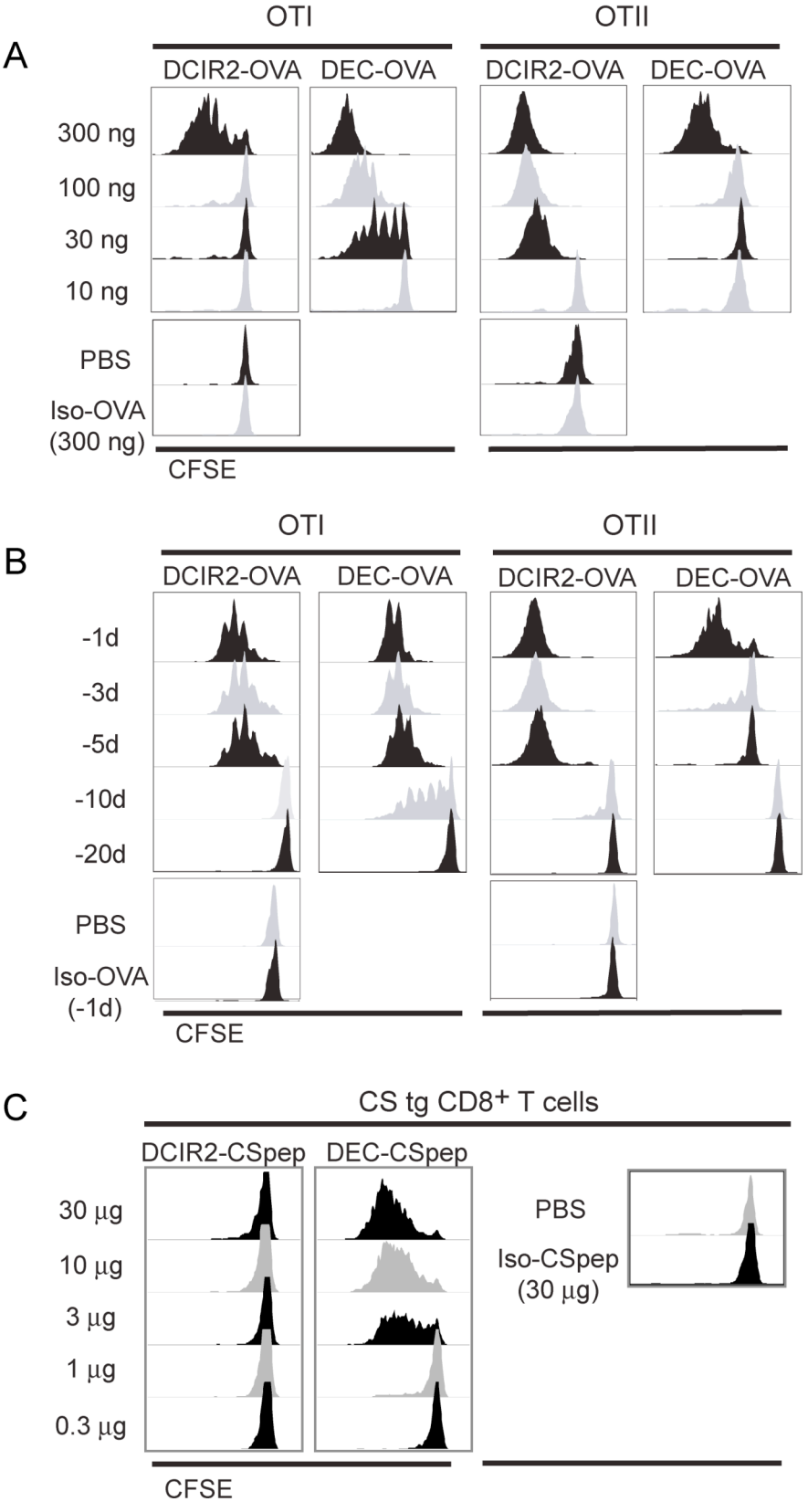
Antigen targeting to $CD8^-$ DCs enabled the direct comparison of antigen processing and presentation capacity by the two major DC subsets: $CD8^+DEC\text{-}205^+$ and $CD8^-DCIR2^+$. To assess antigen presentation *in vivo*, OVA specific $CD8^+$ OTI and $CD4^+$ OTII T cells were labeled with 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE), a reporter dye for cell division, transferred to a new host and monitored after antigen administration. Both anti-DCIR2-OVA and anti-DEC-OVA elicited MHCII and MHCI-restricted T cell responses with only 300 ng of chimeric antibody administration (Fig 2A, *by Diana Dudziak*). *In vivo* dose responses analysis showed that anti-DCIR2-OVA was 10 fold less efficient at MHCI presentation (OTI T cell proliferation) than anti-DEC-OVA. Conversely, for MHCII presentation (OTII T cell proliferation), anti-DCIR2-OVA was 10 fold more effective than anti-DEC-OVA (Fig. 2A, *by Diana Dudziak*). Antigen presentation after a single dose of chimeric antibody was long lasting; anti-DEC-OVA elicited OTI proliferation when T cell transfer occurred up to 10 days after antigen administration and anti-DCIR2-OVA elicited OTII proliferation when T cells were transferred up to 5 days after antigen administration (Fig. 2B).

The superiority of DEC-205 targeting to $CD8^+$ DCs in eliciting MHCI-restricted responses was also observed in BALB/c mice. Chimeric anti-DEC antibodies carrying a $CD8^+$ T cell epitope from the circumsporozoite protein (CS) of *Plasmodium yoelii* (anti-DEC-CSpep) were at least 10 fold better than anti-DCIR2-CSpep at triggering T cell division of transferred CS-specific $CD8^+$ T cells (Fig. 2C). These results combined show that antigen delivery with anti-DCIR2 to $CD8^-$ DCs elicits better activation of $CD4^+$ T cells, while antigen delivery with anti-DEC to $CD8^+$ DCs results in more efficient activation of $CD8^+$ T cells.

Antibodies that recognize peptide-MHC complexes can be used to directly measure antigen presentation. For MHCI presentation, the 25D1 antibody was described to recognize the OVA peptide SIINFEKL in H-$2K^b$ molecules (Porgador et al., 1997). Despite several attempts using the 25D1 antibody no significant signal in DCs after anti-DEC-OVA administration was detected, neither *in vivo* nor *in vitro*. For monitoring peptide-MHCII complexes, there is an antibody that detects the Y-Ae peptide displayed by IA^b molecules (Murphy et al., 1989). However no chimeric antibodies (anti-DCIR2 or anti-DEC) carrying the Y-Ae peptide could be produced; the antibodies where unstable, and purified antibodies were devoid of the Y-Ae peptide. I was finally successful when producing chimeric antibodies carrying a HEL peptide (Hawiger et al., 2001) and assessing IA^k-HEL peptide formation using the Aw3.18 antibody (Dadaglio et al., 1997) in B10.BR mice.

Figure 2. CD4 and CD8 T cell responses to antigens targeted *in vivo* to different DC subsets. A, Histograms show proliferation as measured by CFSE dye dilution of transferred OTI (left) or OTII (right) T cells, after injection of varying amounts of anti-DCIR2-OVA, anti-DEC-OVA or control Iso-OVA antibodies. B, as in A, but T cells were transferred 1, 3, 5, 10 or 20 days after injection of 3 μg of DCIR2-OVA, anti-DEC-OVA or Iso-OVA. C, Histograms show proliferation of transferred CS-specific $CD8^+$ T cells after injection of varying amounts of anti-DCIR2-CSpep, anti-DEC-CSpep or control Iso-CSpep antibodies into BALB/c mice. Data is representative of at least 2 independent experiments.

Figure 2


A
OTI
OTII
DCIR2-OVA
DEC-OVA
DCIR2-OVA
DEC-OVA
300 ng
100 ng
30 ng
10 ng
PBS
Iso-OVA
(300 ng)
CFSE
B
OTI
OTII
DCIR2-OVA
DEC-OVA
DCIR2-OVA
DEC-OVA
-1d
-3d
-5d
-10d
-20d
PBS
Iso-OVA
(-1d)
CFSE
C
CS tg CD8+ T cells
DCIR2-CSpep
DEC-CSpep
30 µg
10 µg
3 µg
1 µg
0.3 µg
PBS
Iso-CSpep
(30 µg)
CFSE

$CD8^+$ DCs showed small amounts of surface MHCII-HEL at 3 hours after injection of anti-DEC-HEL, but after 24 hours it was below detection (Fig. 3A). In contrast, $CD8^-$ DCs targeted with anti-DCIR2-HEL displayed high levels of MHCII-HEL after 3 hours, and it remained high for 2 days (Fig. 3A). Antigen presentation was independent of DC activation, since neither DEC or DCIR2 targeting induced changes in expression of CD40, CD69, CD80, CD86 or MHCII (Dudziak et al., 2007). Moreover, all chimeric antibodies produced had no detectable levels of LPS and similar results were obtained in LPS-insensitive C3H/HeJ mice, which have a spontaneous mutation in TLR4 (Fig. 3B).

Targeting antigens to DCs with anti-DEC or anti-DCIR2 chimeric antibodies in the steady state leads to tolerogenic antigen presentation, whereas concomitant administration of DC maturation stimuli (such as anti-CD40 and polyinosinic :polycytidylic acid) promotes T cell activation and immunity (Bonifaz et al., 2002; Bonifaz et al., 2004; Boscardin et al., 2006; Dudziak et al., 2007; Hawiger et al., 2001; Hawiger et al., 2004; Soares et al., 2007; Trumpfheller et al., 2006). Concomitant administration of LPS or anti-CD40 with anti-DCIR2-HEL resulted in similar MHCII-HEL display on $CD8^-$ DCs as antigen administration without DC activation (Fig. 4A). Interestingly, concomitant administration of LPS with anti-DEC-HEL promoted a slight increased MHCII-HEL display (Fig. 4A). LPS administration increased DEC-205 expression in DCs (not shown here) and had a more significant impact than anti-CD40 to induce DC maturation (Fig. 4B and C).

Figure 3. MHCII-peptide complex formation on DCs after antigen targeting *in vivo* with anti-DCIR2 or anti-DEC-205 antibodies. A, Histograms show MHCII-HEL peptide complexes on $CD8^{+}DEC205^{+}$ and $CD8^{-}DCIR2^{+}$ DCs, 30 min, 3 hours, and 1 or 2 days after intravenous injection of 10 μg anti-DCIR2-HEL, anti-DEC-HEL or control Iso-HEL antibodies in B10.BR mice. B, as in A, but in C3H/HeJ, LPS-insensitive, mice. Data is representative of at least 2 independent experiments.

Figure 3

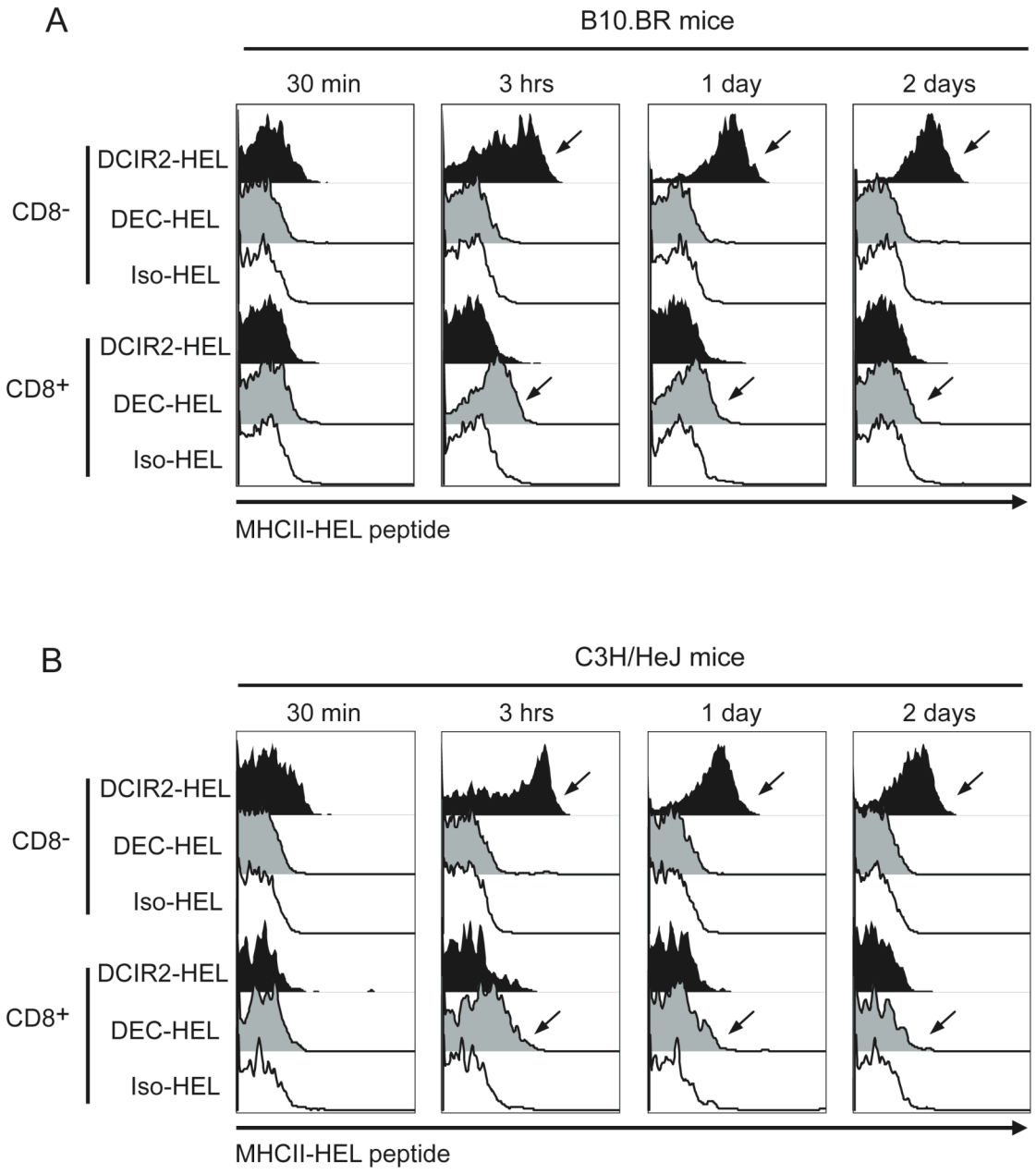
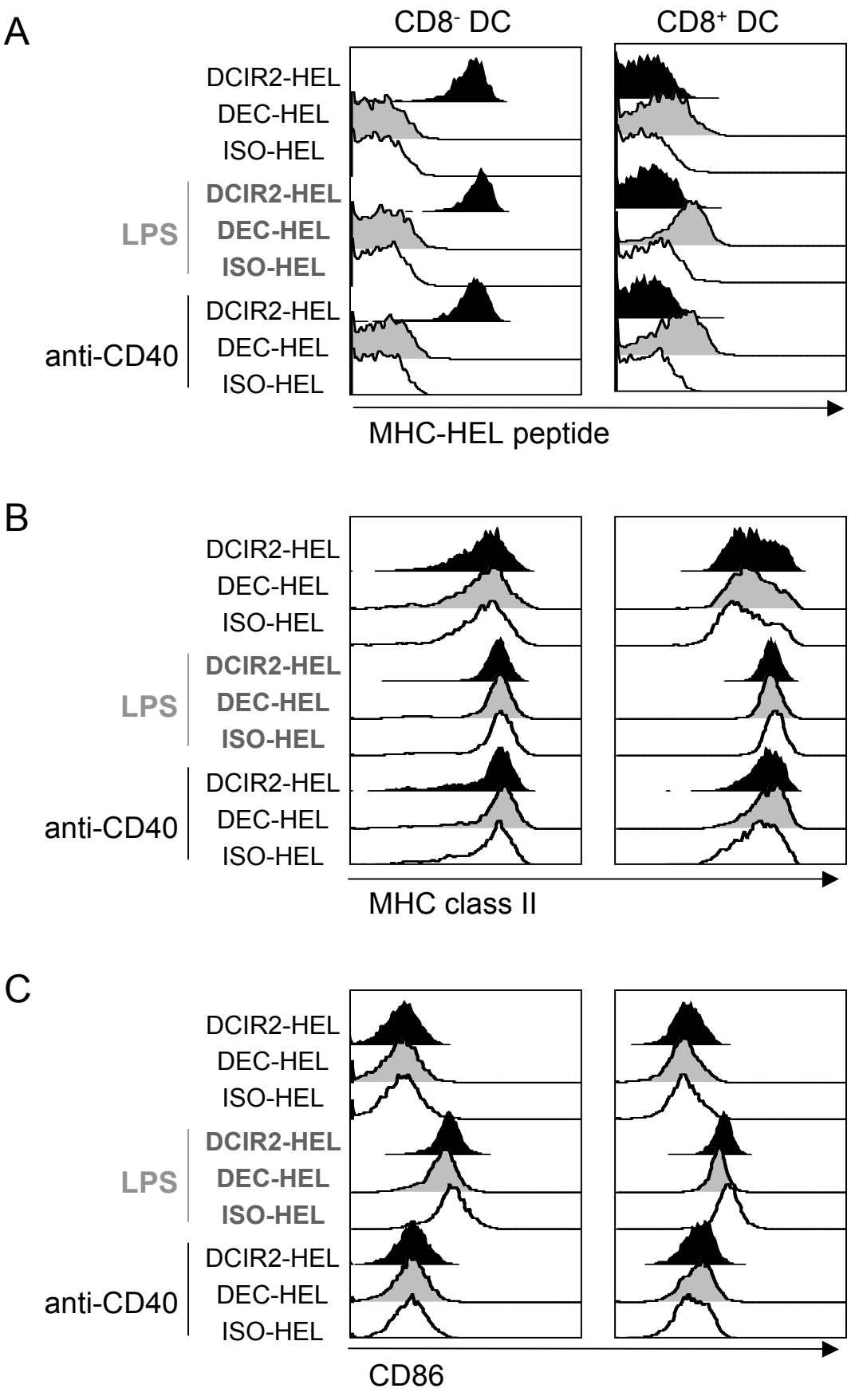

A
B10.BR mice
30 min
3 hrs
1 day
2 days
CD8-
DCIR2-HEL
DEC-HEL
Iso-HEL
CD8+
DCIR2-HEL
DEC-HEL
Iso-HEL
MHCII-HEL peptide
B
C3H/HeJ mice
30 min
3 hrs
1 day
2 days
CD8-
DCIR2-HEL
DEC-HEL
Iso-HEL
CD8+
DCIR2-HEL
DEC-HEL
Iso-HEL
MHCII-HEL peptide

Figure 4. MHCII-peptide complex formation and DC activation after antigen targeting *in vivo* with maturation stimuli. A, Histograms show MHCII-HEL peptide complexes on $CD8^{+}DEC205^{+}$ and $CD8^{-}DCIR2^{+}$ DCs, 6 hours after intravenous injection of 10 μg anti-DCIR2-HEL, anti-DEC-HEL or control Iso-HEL. Where indicated, LPS and anti-CD40 where co-injected with chimeric antibodies. B, as in A, but histograms show MHCII expression. C, as in A but histograms show CD86 expression. Data is representative of at least 2 independent experiments.

Figure 4


A
CD8- DC
CD8+ DC
DCIR2-HEL
DEC-HEL
ISO-HEL
LPS
DCIR2-HEL
DEC-HEL
ISO-HEL
anti-CD40
DCIR2-HEL
DEC-HEL
ISO-HEL
MHC-HEL peptide
B
DCIR2-HEL
DEC-HEL
ISO-HEL
LPS
DCIR2-HEL
DEC-HEL
ISO-HEL
anti-CD40
DCIR2-HEL
DEC-HEL
ISO-HEL
MHC class II
C
DCIR2-HEL
DEC-HEL
ISO-HEL
LPS
DCIR2-HEL
DEC-HEL
ISO-HEL
anti-CD40
DCIR2-HEL
DEC-HEL
ISO-HEL
CD86

In conclusion, antigens delivered by anti-DCIR2 antibodies to $CD8^-$ DCs are processed and transferred to the cell surface as MHCII-peptide complexes more efficiently than antigens delivered by anti-DEC to $CD8^+$ DCs, independent of DC activation status. To determine whether the differences in antigen processing between the two DC subsets were due to the receptor targeted or to cell-intrinsic differences, transgenic mice expressing the human DEC-205 receptor (hDEC) on both DC subsets (CD11c-hDEC mice) were produced (C. Cheong, H-W. Lee and C.G. Park). CD11c-hDEC mice show position-effect variegation, but hDEC is equally expressed and variegated in both $CD8^+$ and $CD8^-$ DCs (Fig. 5A). Anti-hDEC-HEL antibodies, that do not cross-react with endogenous mouse DEC-205 molecules, were cloned (C. Trumpfheller) and produced to target both DC subsets in hDECxB10.BR mice. $CD8^-$ DCs showed significantly higher levels of MHCII-HEL than $CD8^+$ DCs after anti-hDEC-HEL administration (Fig. 5B). Thus cell intrinsic differences determine the superiority of $CD8^-$ DCs over $CD8^+$ DCs for MHCII presentation.

For unknown reasons, no transgenic mice expressing DCIR2 under the control of the CD11c promoter could be obtained to perform reciprocal experiments. Thus, to further compare antigen presentation by anti-DEC and anti-DCIR2 antigen targeting in the same cell, GM-DCs were infected with retroviruses encoding DCIR2 and green fluorescent protein (GFP) (Fig. 6A). Transduced cells expressing DCIR2 were sorted based on GFP expression, then targeted with anti-DEC-OVA or with anti-DCIR2-OVA. LPS was added to induce GM-DC maturation, and MHCII presentation was analyzed by measuring the proliferation of co-cultured OTII T cells. MHCII presentation was equivalent, whether

OVA was captured by DEC-205 or DCIR2 (Fig. 6B). Thus, differential MHCII processing by DC subsets is an intrinsic property of the cells.

In summary, I compared anti-DCIR2-OVA to anti-DEC-OVA targeting and observed that DCIR2 targeting was more effective for MHCII presentation whereas DEC-205 targeting was more efficient for cross-presentation into MHCI. In addition, I was able to demonstrate that $CD8^-$ DCs are better for MHCII antigen processing, independent of the receptor used for antigen capture.

Figure 5. hDEC-205 expression and MHCII-peptide complex formation on DCs after antigen targeting *in vivo* with anti-hDEC-HEL. A, Dot plots show CD8α and hDEC-205 expression on $CD11c^{hi}$ splenocytes on CD11c-hDEC transgenic (left) and B10.BR (right) mice. B, Histograms show MHCII-HEL peptide complexes on $CD8^{+}DEC205^{+}$ and $CD8^{-}DCIR2^{+}$ DCs 30 min, 3 hours, and 1 day after intravenous injection of 30 μg of anti-hDEC-HEL into CD11c-hDEC transgenic ($hDEC^{+}$) or control littermate ($hDEC^{-}$) mice. Data is representative of at least 2 independent experiments.

Figure 5

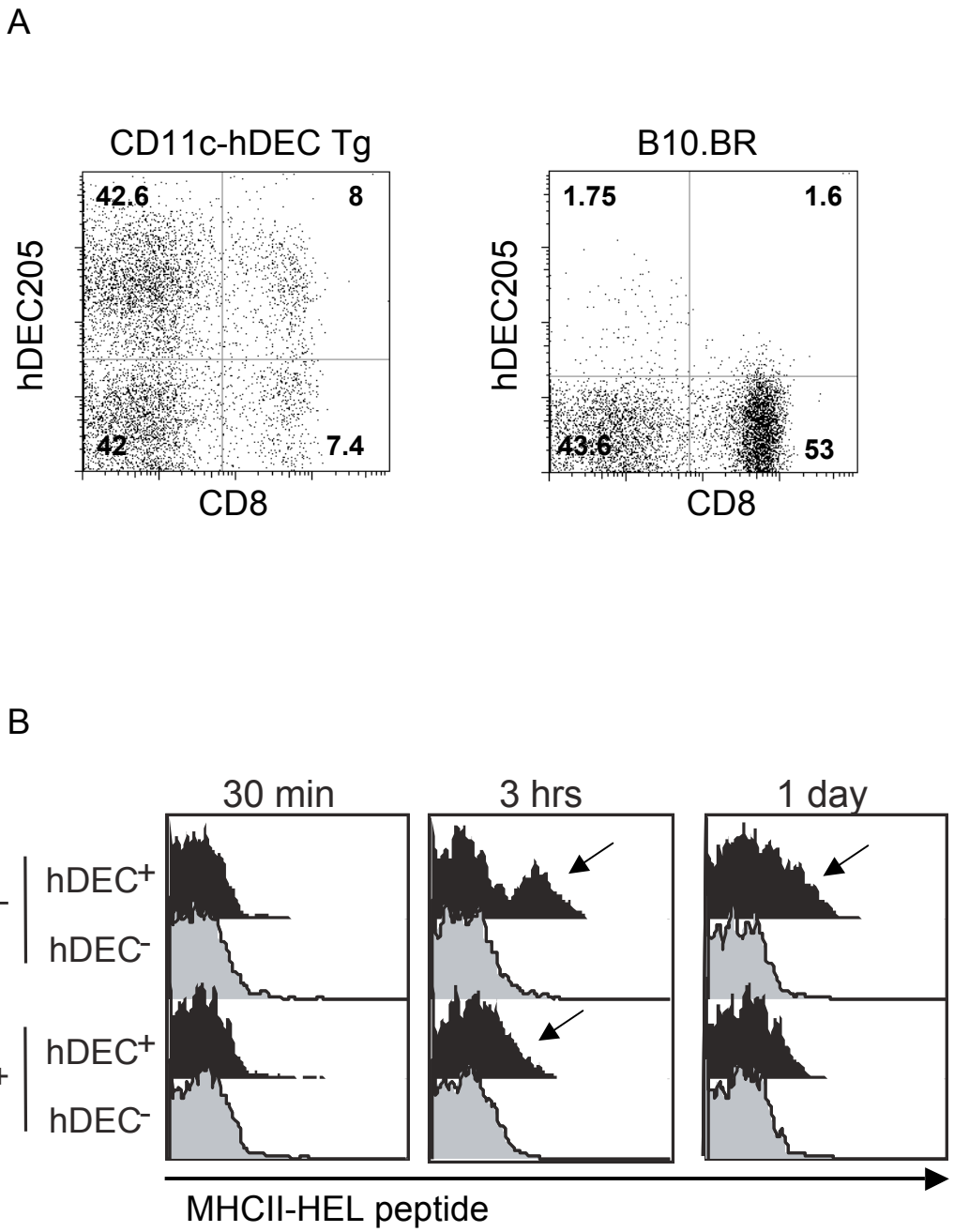
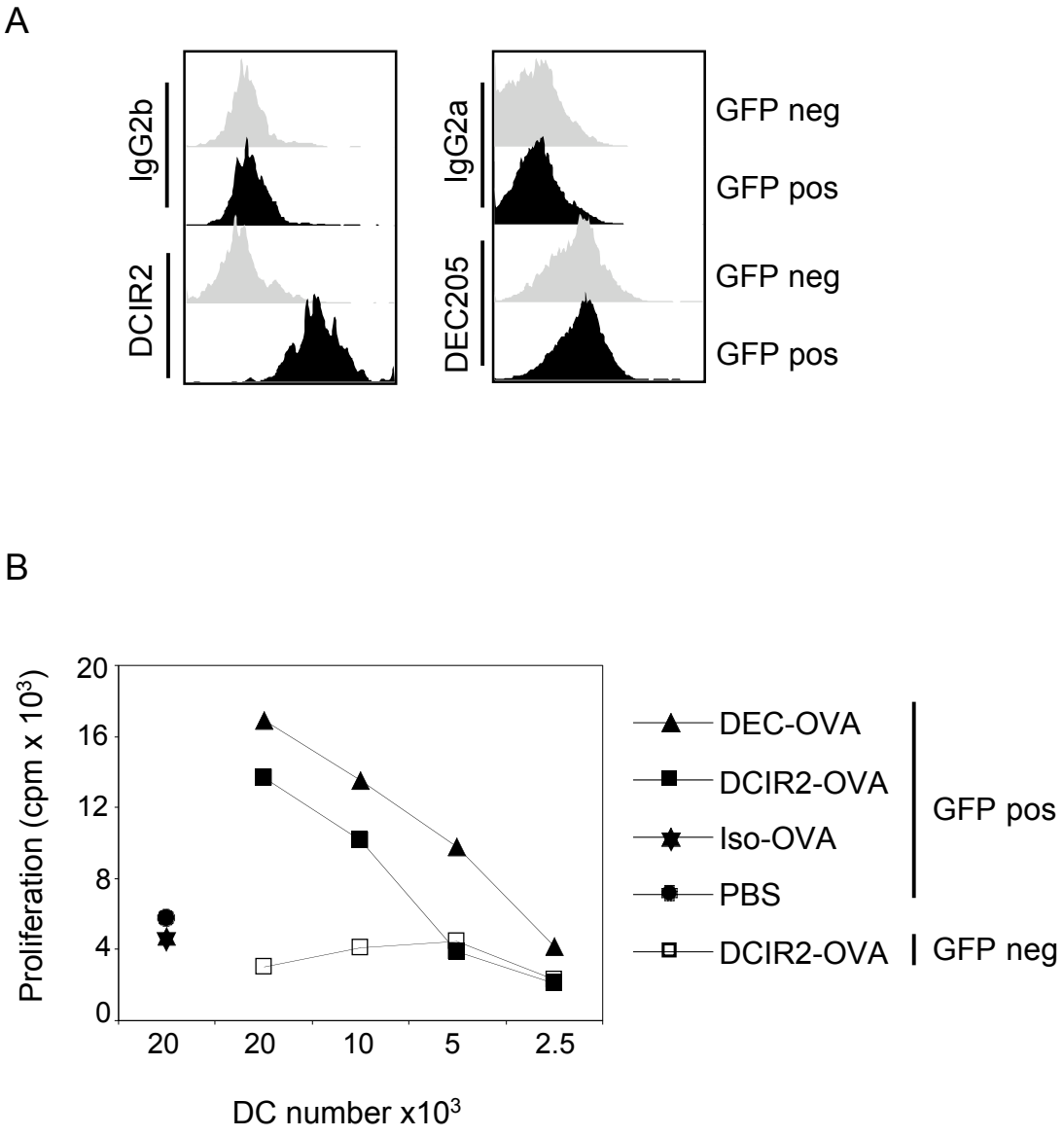

A
CD11c-hDEC Tg
B10.BR
hDEC205
CD8
42.6
8
42
7.4
1.75
1.6
43.6
53
B
30 min
3 hrs
1 day
CD8-
hDEC+
hDEC-
CD8+
hDEC+
hDEC-
MHCII-HEL peptide

Figure 6. Antigen targeting to GM-DCs. A, Histograms show DCIR2 and isotype rat IgG2b (left) and DEC-205 and isotype rat IgG2a (right) staining on day 6 DCIR2-GFP transduced GM-DCs, gated on $CD11c^{+}GFP^{+}$ (GFP pos) and $CD11c^{+}GFP^{-}$ (GFP neg). B, Transduced GM-DCs were sorted into $CD11c^{+}GFP^{+}$ and $CD11c^{+}GFP^{-}$ and incubated with 1 μg /ml anti-DCIR2-OVA, anti-DEC-OVA or Iso-OVA and then LPS. Cells were washed and co-cultured with OTII T cells. Graph shows T cell proliferation measured by [$_3$H]-thymidine incorporation. Data is representative of 2 independent experiments.

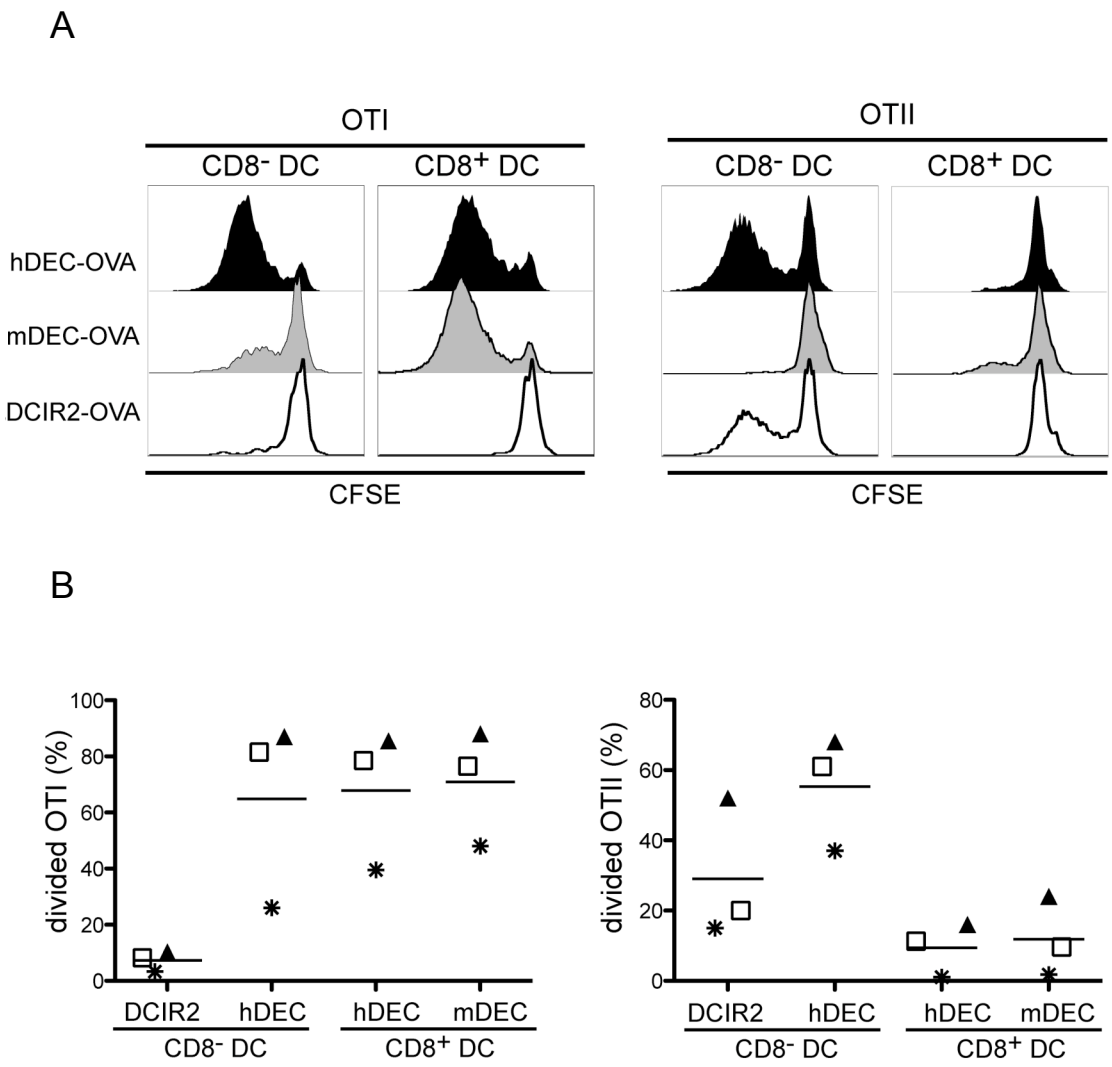
Figure 6


A
IgG2b
DCIR2
IgG2a
DEC205
GFP neg
GFP pos
GFP neg
GFP pos
B
Proliferation (cpm x 10³)
20
16
12
8
4
0
20
20
10
5
2.5
DC number x10³
DEC-OVA
DCIR2-OVA
Iso-OVA
PBS
DCIR2-OVA
GFP pos
GFP neg

I next sought to understand if the differences between $CD8^+$ and $CD8^-$ DCs for MHCI cross-presentation were also due to cell-intrinsic differences. CD11c-hDEC mice were injected with anti-DCIR2-OVA, anti-mouse-DEC-OVA (anti-mDEC-OVA) and anti-hDEC-OVA. hDEC-expressing $CD8^+$ and $CD8^-$ DCs were purified by cell sorting and evaluated for MHCI presentation by co-culture with CFSE labeled OTI T cells. To confirm the previous results on MHCII presentation, sorted DCs were also co-cultured with CFSE labeled OTII T cells. As previously documented, $CD8^-$ DCs targeted *in vivo* with anti-DCIR2-OVA efficiently induced OTII but not OTI proliferation; whereas $CD8^+$ DCs targeted *in vivo* with anti-mDEC-OVA induced robust OTI but modest OTII proliferation (Fig. 7). Furthermore, when both DC subsets were targeted with anti-hDEC-OVA, $CD8^-$ DCs were more effective at stimulating OTII proliferation than $CD8^+$ DCs from the same mice (Fig. 7). In contrast, both $CD8^-$ and $CD8^+$ DCs targeted *in vivo* with anti–hDEC-OVA elicited similarly robust levels of OTI proliferation (Fig. 7). These results indicate that both DC subsets have the same intrinsic potential to cross-present antigens when the antigen is delivered by DEC-205.

Figure 7. hDEC-205-mediated endocytosis *in vivo* promotes cross-presentation by both $CD8^-$ and $CD8^+$ DC subsets. A, Representative histograms show proliferation as measured by CFSE dye dilution of OTI (left) and OTII (right) T cells upon culture with $5x10^3$ $CD8^-$ or $CD8^+$ DCs isolated from CD11c-hDEC transgenic mice injected with anti-hDEC-OVA, anti-mDEC-OVA or anti-DCIR2-OVA, as indicated. B, Summary of 3 independent experiments as in A, where panels show the percentage of divided, CFSE low OTI (left) and OTII (right) cells. Each symbol indicates independent experiments and represents the average of duplicate measurements.

Figure 7


A
OTI
OTII
CD8- DC
CD8+ DC
CD8- DC
CD8+ DC
hDEC-OVA
mDEC-OVA
DCIR2-OVA
CFSE
CFSE
B
divided OTI (%)
100
80
60
40
20
0
DCIR2
hDEC
hDEC
mDEC
CD8- DC
CD8+ DC
divided OTII (%)
80
60
40
20
0
DCIR2
hDEC
hDEC
mDEC
CD8- DC
CD8+ DC

Chapter 4

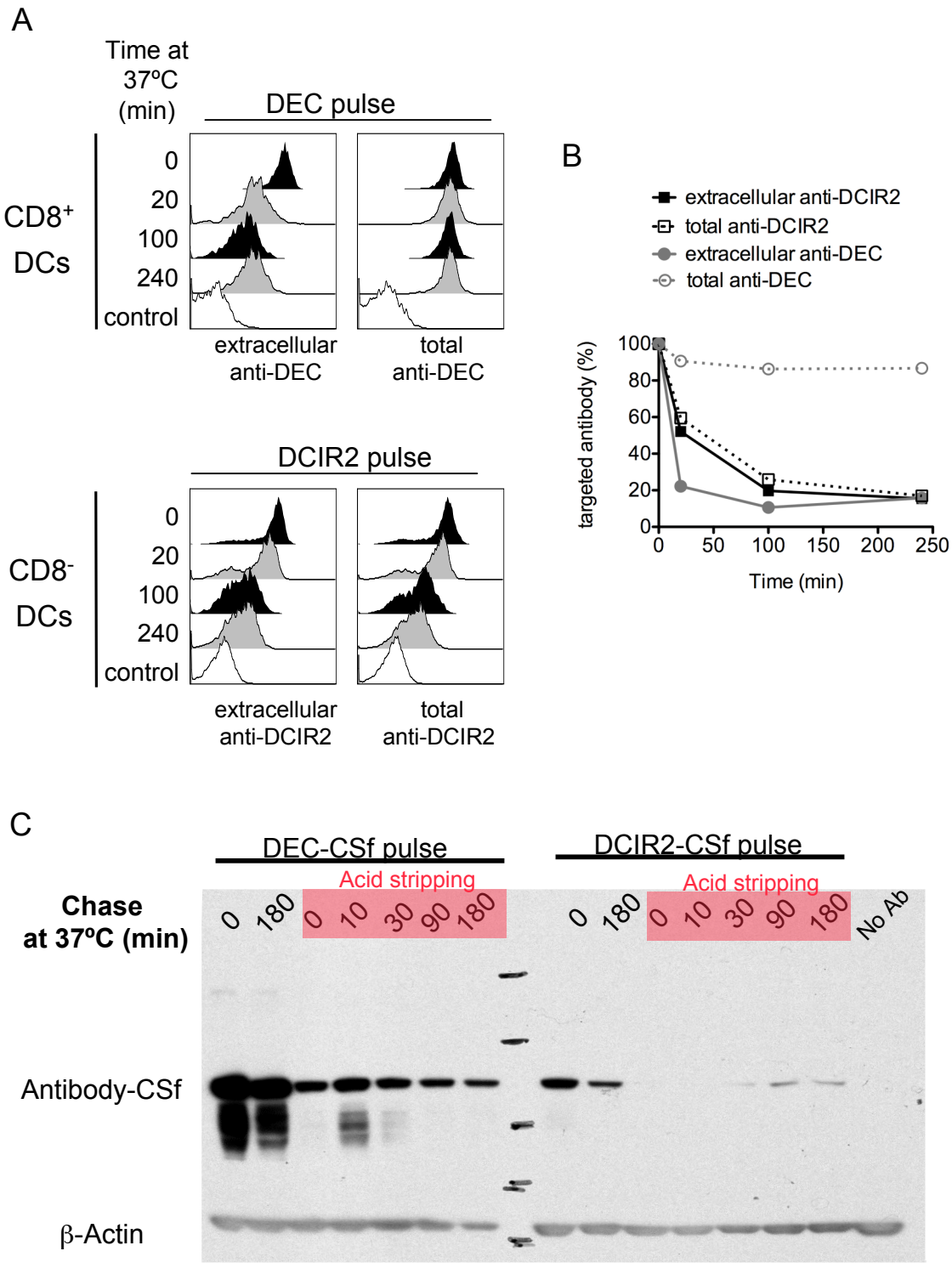
Results Part II

Internalization of anti-DCIR2 antibodies was slower than anti-DEC antibodies (Dudziak et al., 2007). I confirmed those results with *in vitro* pulse-chase experiments (Fig. 8). Splenocytes were incubated with anti-DCIR2 or anti-DEC antibodies on ice, excess antibodies were washed, and cells were incubated at 37°C for 20 to 240 minutes (Fig. 8A and B). Internalization of targeting antibodies was monitored by extracellular anti-Rat antibody detection. In addition, cells were fixed and permeabilized to detect total cell-associated anti-DCIR2 or anti-DEC antibodies. For anti-DEC antibodies efficient internalization was confirmed as extracellular anti-Rat detection decreased over time at 37°C (Fig. 8A upper panels and 8B). The total amount of anti-DEC antibodies associated with $CD8^+$ DCs did not decrease over time (Fig. 8A upper panels and 8B). Conversely, the total amount of anti-DCIR2 antibodies associated with $CD8^-$ DCs correlated to the antibodies that remained in the plasma membrane, and total cell associated antibody sharply decreased over time (Fig. 8A lower panels and 8B).

Similar results were obtained by Western blot detection of targeted anti-DEC and anti-DCIR2 antibodies carrying the circumsporozoite protein from *Plasmodium falciparum* (CSf). Enriched $CD11c^+$ splenocytes were incubated on ice with anti-DCIR2-CSf or anti-DEC-CSf antibodies (kindly provided by S. Boscardin), and incubated for 10 to 180 minutes at 37°C. Incubation with pH 3.6 buffer solution was used to remove antibodies

Figure 8. Internalization of anti-DEC-205 and anti-DCIR2. A and B, Splenocytes were incubated on ice, with either anti-DEC or anti-DCIR2 targeting antibodies, washed and incubated at 37°C for various lengths of time. Control cells were not incubated with targeting antibodies A, Histograms show detection of the targeting antibodies on the cell surface of the respective DCs (extracellular staining, left panels) or after fixation and permeabilization (total staining, right panels). B, Graph is a summary of A, where mean fluorescence values at time 0 were normalized to 100%. C. $CD11c^+$ enriched splenocytes were incubated with anti-DEC-CSf or anti-DCIR2-CSf on ice, washed and incubated at 37°C for various times. Indicated cell samples were treated with pH 3.6 buffer to remove extracellular antibodies. Protein extracts from cell samples were obtained and probed for CSf and β-actin by Western blot. Data is representative of at least 2 independent experiments.

Figure 8


A
Time at 37ºC (min)
DEC pulse
CD8+ DCs
0
20
100
240
control
extracellular anti-DEC
total anti-DEC
DCIR2 pulse
CD8- DCs
0
20
100
240
control
extracellular anti-DCIR2
total anti-DCIR2
B
extracellular anti-DCIR2
total anti-DCIR2
extracellular anti-DEC
total anti-DEC
targeted antibody (%)
100
80
60
40
20
0
0
50
100
150
200
250
Time (min)
C
DEC-CSf pulse
DCIR2-CSf pulse
Acid stripping
Acid stripping
Chase at 37ºC (min)
0
180
0
10
30
90
180
0
180
0
10
30
90
180
No Ab
Antibody-CSf
β-Actin

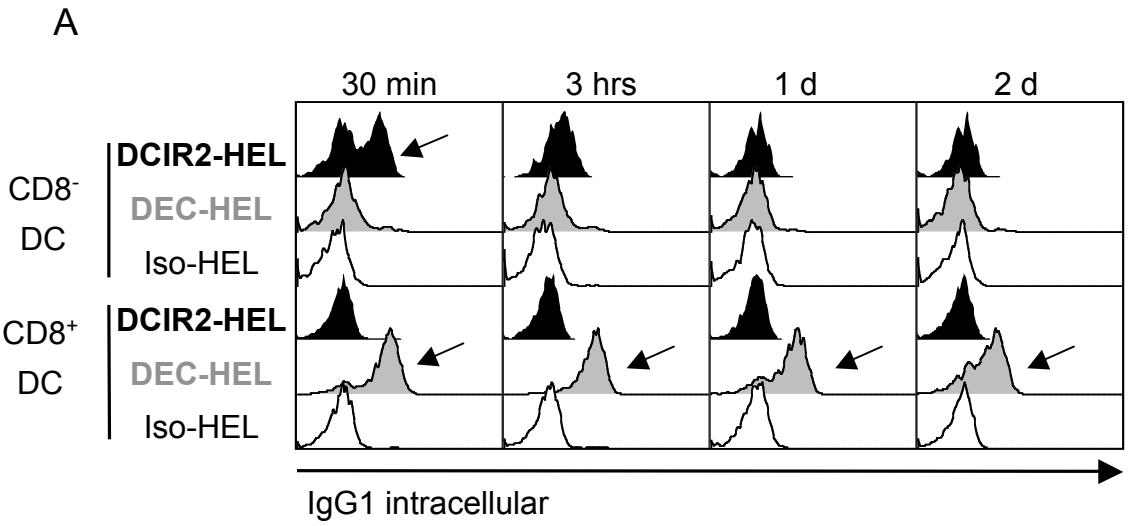
from the plasma membrane and allow visualization of internalized antibodies. Anti-DEC not only delivered more antigen to DCs than anti-DCIR2, but also that internalization was faster and more effective (Fig. 8C). Even though acid stripping was not efficient at removing all anti-DEC-CSf antibodies from the cell surface, accumulation of internalized antibodies could be detected after 10 min. But internalized anti-DCIR2 antibodies could only be detected after 30 minutes (Fig 8C).

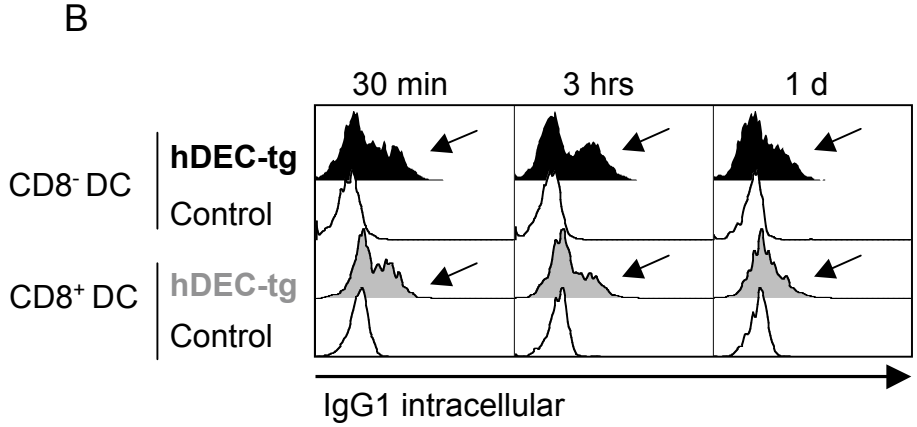
To analyze the fate of anti-DEC and anti-DCIR2 antibodies after internalization *in vivo*, mice were injected with anti-DCIR2-HEL, anti-mDEC-HEL and control isotype-HEL and internalized antibodies were detected on permeabilized splenocytes. 30 minutes after administration both anti-DCIR2-HEL and anti-DEC-HEL could be detected inside $CD8^-$ and $CD8^+$ DCs, respectively (Fig 9A). However, anti-DCIR-HEL antibodies could not be detected after 3 hours, while internalized anti-DEC-HEL antibodies could still be detected up to two days after administration (Fig 9A).

To determine if the difference in the persistence of internalized antibodies was due to the receptor targeted (DEC or DCIR2) or cell intrinsic differences between DC subsets, similar experiments were performed in CD11c-hDEC transgenic mice. Anti-hDEC-HEL antibodies were detected in equivalent amounts inside both $CD8^-$ and $CD8^+$ DCs (Fig. 9B). In conclusion, anti-DEC antibodies are rapidly internalized and can be detected inside DCs for prolonged periods (at least 2 days). On the other hand, anti-DCIR2 antibodies are internalized more slowly, and their detection inside DCs is less robust and only possible at early time-points (30 min). Those features are related to the receptor

Figure 9. Persistence of internalized anti-DEC-205 antibodies *in vivo.* A, Histograms show internalized targeted antibodies on $CD8^{+}DEC205^{+}$ and $CD8^{-}DCIR2^{+}$ DCs, 30 min, 3 hours, and 1 or 2 days after intravenous injection of 10 μg anti-DCIR2-HEL, anti-DEC-HEL or control Iso-HEL antibodies into B10.BR mice. Internalized antibodies were detected by blocking extracellular antibodies with unlabeled anti-mouse IgG1, then splenocytes were fixed and permeabilized and intracellular targeting antibodies were detected with anti-mouse IgG1 FITC. B, as in A, but histograms show internalized targeted antibodies on $CD8^{+}DEC205^{+}$ and $CD8^{-}DCIR2^{+}$ DCs after intravenous injection of 30 μg of anti-hDEC-HEL into CD11c-hDEC transgenic mice or negative littermates (control). Data is representative of at least 2 independent experiments.

Figure 9


A
30 min
3 hrs
1 d
2 d
CD8- DC
DCIR2-HEL
DEC-HEL
Iso-HEL
CD8+ DC
DCIR2-HEL
DEC-HEL
Iso-HEL
IgG1 intracellular


B
30 min
3 hrs
1 d
CD8- DC
hDEC-tg
Control
CD8+ DC
hDEC-tg
Control
IgG1 intracellular

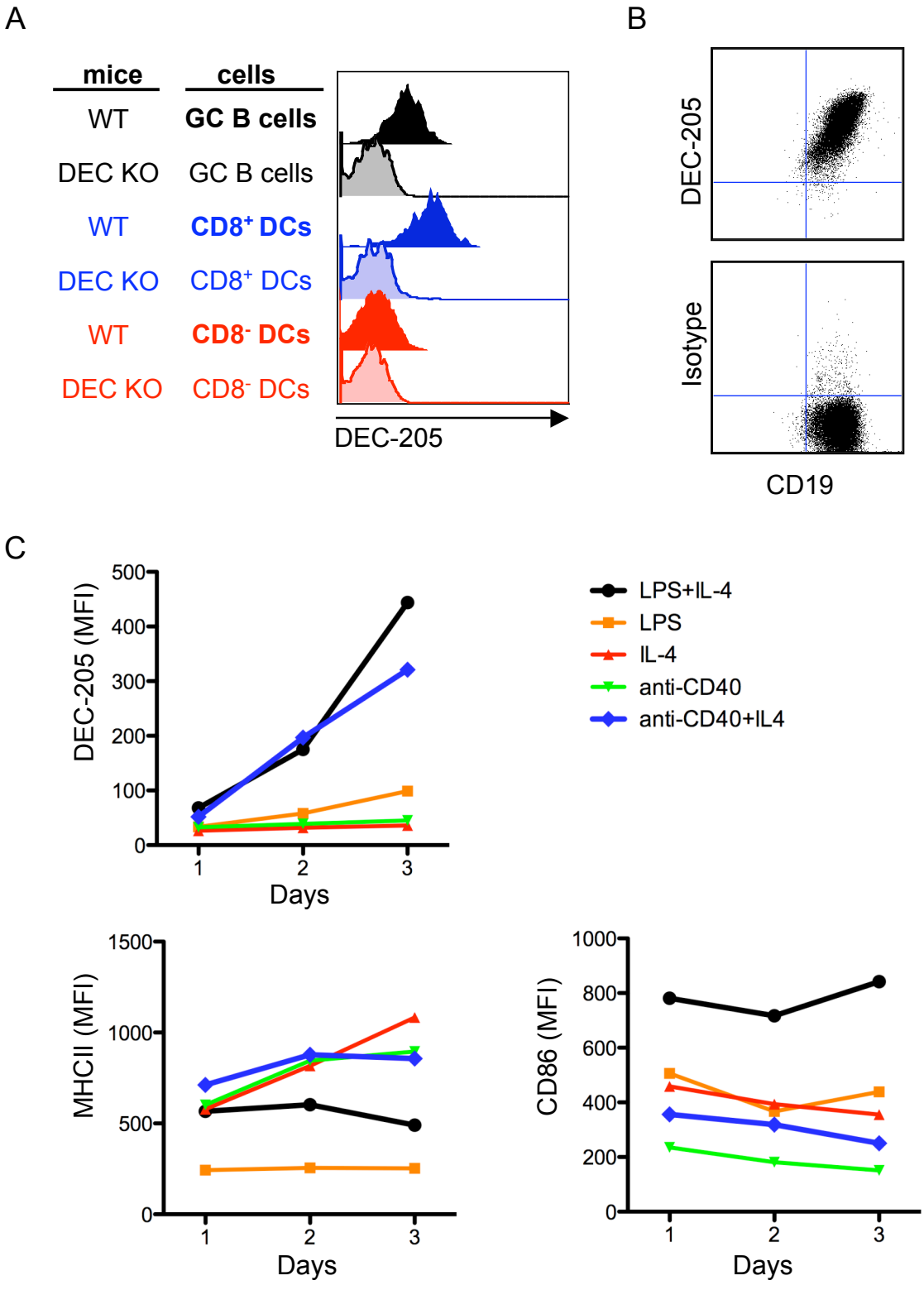
used for internalization. Supporting these conclusions, similar results were also obtained in a DC cell line transduced with DEC and DCIR2 (data not shown).

When antigen was delivered by DEC-mediated endocytosis, both $CD8^+$ and $CD8^-$ DCs were equivalent in cross-presentation. However, distinct internalization kinetics between DEC and DCIR2 impose certain difficulty in comparing differences in the intracellular route of the targeted antibodies. Thus to investigate whether the DEC-205 molecule direct antigens to a special intracellular route favoring cross-presentation, I started searching for alternative models.

Even though DEC-205 is highly expressed by some subsets of DCs, it is not restricted to DCs; thymic epithelium and B cells, for example, also express DEC-205. However DEC-205 expression in splenic B cells was evaluated to be at least one order of magnitude lower than in DCs, and it did not increase upon B cell culture with LPS, anti-CD40 or the combination of anti-IgM and IL-4 (Inaba et al., 1995). I repeated this analysis and found that germinal center B cells (Fas^+, GL-7^+) express DEC-205 at levels comparable to DCs (Fig. 10A). Germinal center B cells constitute a negligible population in naïve mice and therefore can be easily overlooked. I also analyzed *in vitro* stimulation conditions that would promote DEC-205 expression in B cells. The combination of LPS and IL-4 or anti-CD40 and IL-4 prompted high levels of DEC-205 expression in cultured B cells (Fig. 10B and C). Interestingly, there was no direct correlation between the level of DEC-205 expression and activation markers such as MHCII or CD86 (Fig. 10C).

Figure 10. DEC-205 expression in B cells. A, Histograms show DEC-205 expression on germinal center B cells ($CD19^+$ Fas^+ $GL\text{-}7^+$), $CD8^+$ and $CD8^-$ DCs from WT or DEC-$205^{-/-}$ mice. B, Dot-plots show CD19 and DEC-205 or isotype staining on B cells cultured with LPS and IL-4 for 3 days. C, Graphs show expression of DEC-205, MHCII and CD86 on B cells cultured with different stimuli for 1, 2 or 3 days, as indicated. Data is representative of at least 2 independent experiments.

Figure 10


A
mice
cells
WT
GC B cells
DEC KO
GC B cells
WT
CD8+ DCs
DEC KO
CD8+ DCs
WT
CD8- DCs
DEC KO
CD8- DCs
DEC-205
B
DEC-205
Isotype
CD19
C
DEC-205 (MFI)
500
400
300
200
100
0
1
2
3
Days
LPS+IL-4
LPS
IL-4
anti-CD40
anti-CD40+IL4
MHCII (MFI)
1500
1000
500
0
1
2
3
Days
CD86 (MFI)
1000
800
600
400
200
0
1
2
3
Days

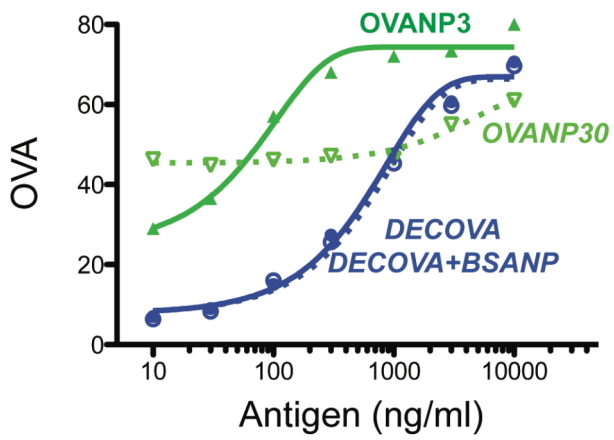
B cells are also professional antigen presenting cells and they rely on their B-cell receptor (BCR) for antigen capture. The BCR consists of a membrane-associated immunoglobulin (Ig), associated with invariant Igα-Igβ heterodimers. Cross-linking of the BCR triggers its internalization into antigen processing compartments and also initiates a signaling cascade that leads to B cell activation, enabling efficient antigen presentation. To compare DEC-mediated to BCR-mediated antigen capture for antigen presentation, B cells were isolated from B1-8^{hi} mice, specific for NP (4-hydroxy-3-nitrophenil). B cells were cultured for 2 days in LPS and IL-4 and then pulsed with different concentration of either anti-DEC-OVA or OVA-NP. Excess antigen was washed off, and B cells were co-cultured with CFSE labeled OVA-specific OTI or OTII T cells. When the relative amount of antigen uptake is taken into account, similar results were obtained for MHCII presentation for antigens targeted to the BCR instead of DEC-205 (Fig. 11). However, antigen captured by DEC-205 was approximately three times more efficient than the BCR for cross-presentation (Fig. 11). This difference is not dependent on BCR cross-linking, since anti-DEC-OVA together with BSA-NP was equivalent to anti-DEC-OVA alone, and OVA-NP3 was similar to OVA-NP30.

The cytoplasmic tails of receptors contain amino acid motifs that are responsible for interacting with different adaptors and determine their intracellular sorting (Bonifacino, 2003). Based on antigen presentation results, the intracellular sorting of DEC-205 might be important for delivering antigens to compartments that enhance cross-presentation. Hence, undertaking a genetic approach, the cross-presentation ability of different DEC-mutants (Table 1) where the cytoplasmic portion was modified were compared. Different

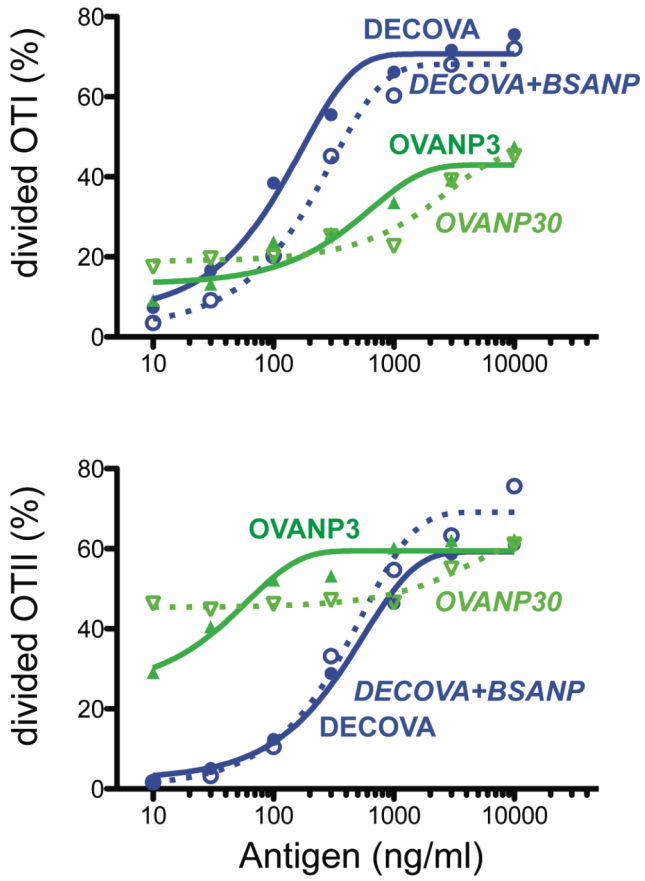
Figure 11. DEC-205-mediated endocytosis promotes cross-presentation in B cells. NP-specific B cells were isolated from B1-8^{hi} transgenic mice and stimulated with LPS and IL-4 for 50-60 hours. A, The Y-axis shows relative cell-associated OVA, after targeting with the indicated concentrations of anti-DEC-OVA, or anti-DEC-OVA and BSA-NP, or OVA-NP3, or OVA-NP30 on the X-axis. Maximum cell-associated OVA was normalized to 100 in each experiment. B, Activation and proliferation of OTI T cells (upper panel) and OTII T cells (lower panel) in response to OVA containing B cells. The Y-axis shows percentage of divided T cells. A and B represent pooled data from 3 independent experiments.

Figure 11

A


OVANP3
OVANP30
DECOVA
DECOVA+BSANP
OVA
80
60
40
20
0
10
100
1000
10000
Antigen (ng/ml)

B


DECOVA
DECOVA+BSANP
OVANP3
OVANP30
divided OTI (%)
OVANP3
OVANP30
DECOVA+BSANP
DECOVA
divided OTII (%)
80
60
40
20
0
10
100
1000
10000
Antigen (ng/ml)

DEC-205 mutants with deletions in the C-terminal cytoplasmic tail were designed. For example: DEC-medium lacking the acidic cluster "EDE" that had been implicated with lysossomal targeting (Mahnke et al., 2000); and DEC-short lacking the "EDE" motif and the "FSSVRY" motif (implicated in coated pit localization) (Chen et al., 1990; Collawn et al., 1990; Paccaud et al., 1993). In addition, mutant DEC-205 molecules containing substitutions of three consecutive amino acids to alanines were also designed.

Table 1: Amino acid sequence of the cytoplasmic tail of wild type DEC-205 and of engineered DEC-mutants

Receptor	cytoplasmic tail
DEC_WT	^{TM}QRSHIRWTG<u>FSSVRY</u>EHGTN<u>**EDE**</u>VMLPSFHD^{-C}
DEC_medEDEVMLP	^{TM}QRSHIRWTG<u>FSSVRY</u>EHGTN<u>**EDE**</u>VMLP^{-C}
DEC_med+EDE	^{TM}QRSHIRWTG<u>FSSVRY</u>EHGTN<u>**EDE**</u>$^{-C}$
DEC_medium	^{TM}QRSHIRWTG<u>FSSVRY</u>EHG^{-C}
DEC_short	^{TM}QRSHIRWTG^{-C}
DEC_VRYtoAAA	^{TM}QRSHIRWTGFSS**AAA**EHGTN<u>**EDE**</u>VMLPSFHD^{-C}
DEC_EDEtoAAA	^{TM}QRSHIRWTG<u>FSSVRY</u>EHGTN**AAA**VMLPSFHD^{-C}
DEC_VMLtoAAA	^{TM}QRSHIRWTG<u>FSSVRY</u>EHGTN<u>**EDE**</u>**AAA**PSFHD^{-C}

TM: Transmembrane C: C-terminus <u>FSSVRY</u> <u>**EDE**</u>

Mouse DEC-205 molecules (WT and mutant versions) were cloned into retroviral vectors that contained a puromycin resistance gene. Ideally, I wanted to perform experiments using bona fide DCs; however after isolation, DCs do not divide or survive long, and heterologous protein expression is not optimal. Therefore DC cell lines were obtained and transduced. D1 cells (Winzler et al., 1997) were difficult to grow and maintain, and

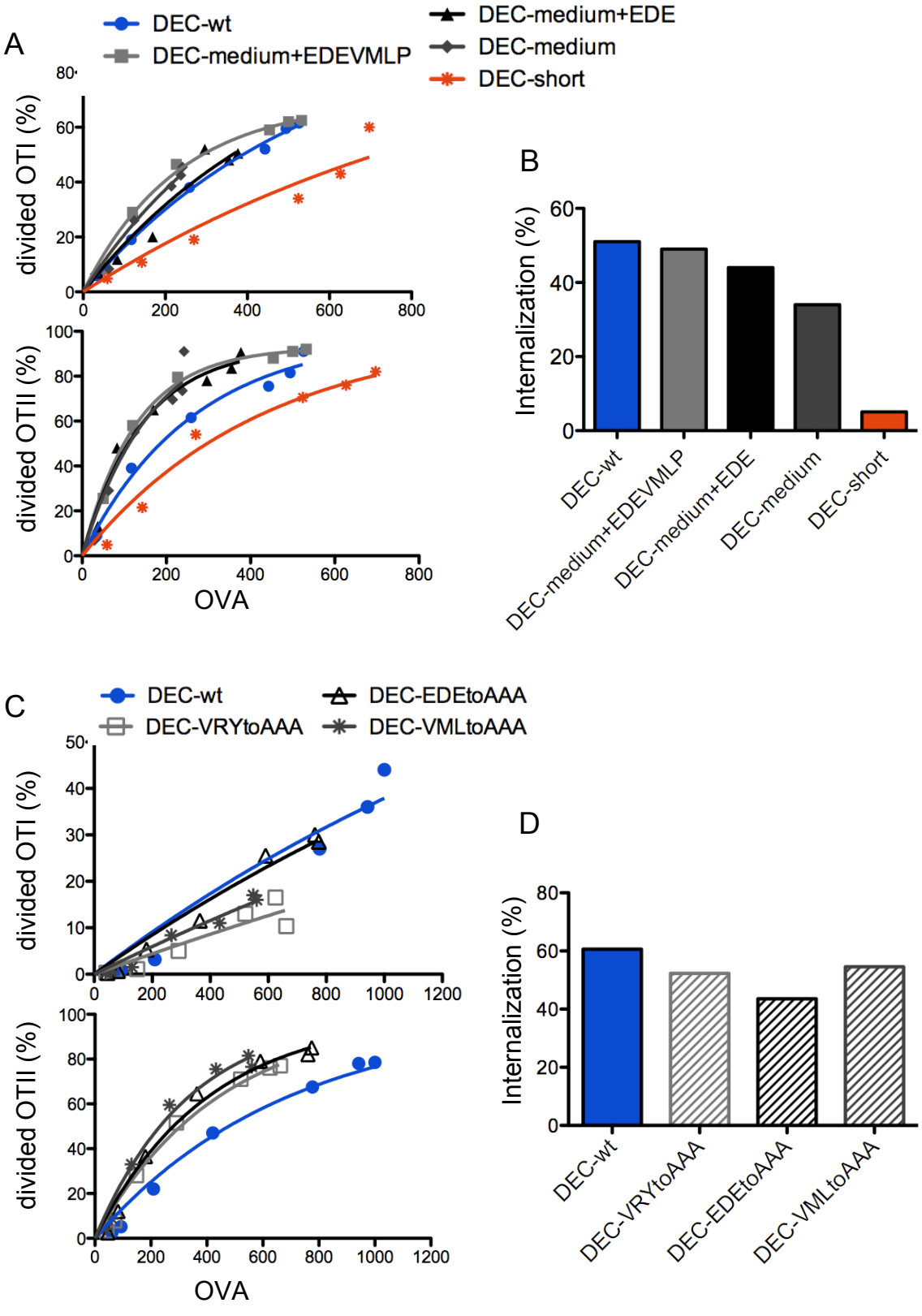
no transduced cell lines could be selected. DC2.4 cells (Shen et al., 1997) could be transduced and selected, but the cells were unable to activate antigen specific $CD4^+$ T cells (even with exogenous peptide loading and additional stimulation with cytokines and TLR ligands). Moreover, DC2.4 cells were not optimal to present exogenous antigens to $CD8^+$ T cells. Hence, I decided to study the role of DEC cytoplasmic tail on transduced primary B cells stimulated with LPS and IL-4.

B cells from DEC-205 deficient mice were transduced with DEC-WT and DEC-mutants. DEC mRNA is 5.2 kilobases, limiting efficient packaging into viruses. However, after puromycin selection, enriched transduced cells could be obtained. Transduced B cells had very different membrane expression levels of the DEC-mutants; therefore it was imperative to measure the amount of OVA bound by each receptor after anti-DEC-OVA targeting. Transduced B cells were pulsed on ice with different concentrations of anti-DEC-OVA, washed and stained with rabbit anti-OVA antibodies to assess the amount of OVA captured. Replica plates of antigen pulsed B cells were co-cultured with CFSE labeled OTI or OTII T cells. To evaluate the efficiency of antigen presentation, corrected for the amount of antigen captured, the results are displayed in the same graph: the amount of captured OVA on the X-axis and T-cell proliferation on the Y-axis (Fig. 12A and C). In addition, DEC-205 endocytosis was measured to evaluate antigen internalization (Fig. 12B and D).

Surprisingly, except for the DEC-short tail, which was severely impaired for internalization (Fig. 12B) and thus showed a reduction in cross-presentation and MHCII presentation (Fig. 12A), none of the DEC mutations assayed significantly impacted antigen processing and presentation for $CD4^+$ or $CD8^+$ T cells (Fig. 12). In conclusion, the amount of antigen captured and internalized seems to be the main factor that controls the level of MHCII presentation or cross-presentation.

Figure 12. Most mutations in the cytoplasmic tail of DEC-205 do not disrupt internalization or antigen presentation. A, Naïve B cells from DEC-205$^{-/-}$ mice were stimulated with LPS and IL-4 and transduced with DEC-wt or DEC-mutants with deletions in the cytoplasmic tail, according to table 1. Enriched transduced cells were pulsed with anti-DEC-OVA (1-10,000 ng/ml). Cell-associated OVA was assessed with rabbit anti-OVA (mean fluorescence intensity on the X-axis), and B cells were co-cultured with OTI T cells (upper panel) or OTII T cells (lower panel). The Y-axis shows percentage of divided T cells in response to OVA containing B cells. B, as in A, but percentage of DEC-205 internalization is shown. B cells were stained on ice with anti-DEC antibodies, followed by incubation at 37°C for 3 hours. Anti-DEC antibodies remaining on the cell surface were detected with anti-Rat staining and the percentage of internalization was calculated. C and D, as in A and B, but DEC-mutants had substitutions of 3 residues in the cytoplasmic tail for 3 alanines, as indicated. Data is representative of at least 2 independent experiments.

Figure 12


A
DEC-wt
DEC-medium+EDEVMLP
DEC-medium+EDE
DEC-medium
DEC-short
divided OTI (%)
divided OTII (%)
OVA
B
Internalization (%)
DEC-wt
DEC-medium+EDEVMLP
DEC-medium+EDE
DEC-medium
DEC-short
C
DEC-wt
DEC-VRYtoAAA
DEC-EDEtoAAA
DEC-VMLtoAAA
divided OTI (%)
divided OTII (%)
OVA
D
Internalization (%)
DEC-wt
DEC-VRYtoAAA
DEC-EDEtoAAA
DEC-VMLtoAAA

Chapter 5

Results Part III

A key factor that impacts antigen presentation of a given peptide is the amount of antigen captured. Yet very few studies on antigen presentation measure antigen uptake. *In vivo*, this caveat can be justified because antigen capture and presentation constitute a continuous process where after internalization the antigen is processed into peptides in order to be loaded onto MHC molecules and presented to T cells. Antigen processing into peptides hampers methods of antigen detection that rely on protein integrity.

The route of antigen capture also impacts antigen presentation: recently it has been show that OVA uptake by the mannose receptor potentiates cross-presentation (Burgdorf et al., 2007). In addition, when antigen is delivered by DEC-mediated endocytosis, both $CD8^+$ and $CD8^-$ DCs are equivalent in cross-presentation (Fig. 7). And in B cells DEC-mediated antigen uptake is more efficient than the BCR for cross-presentation (Fig. 11).

Finally cell intrinsic factors also modulate antigen presentation: $CD8^-$ DCs are better at MHCII presentation than $CD8^+$ DCs, even when antigen is targeted to the same receptor (hDEC) (Fig. 5 and 6)(Dudziak et al., 2007). Although both subsets of DCs express similar levels of hDEC-205 in CD11c-hDEC transgenic mice (Dudziak et al., 2007), differences in antigen presentation may nevertheless be the result of differences in the amounts of antigen captured. To explore this possibility I isolated spleen $CD8^-$ and $CD8^+$

DCs from CD11c-hDEC mice, pulsed them on ice with varying amounts of anti-hDEC-OVA and measured the amount of cell-associated OVA (Fig. 13A, left). Replica plates of pulsed DCs were co-cultured with CFSE labeled OTI or OTII transgenic T cells to measure their ability to present OVA (Fig. 13B, left). As a further control, DCs were pulsed with peptides to measure their antigen presenting activity independent of antigen capture and processing (Fig. 13C, left).

$CD8^-$ and $CD8^+$ splenic DCs captured similar amounts of OVA when pulsed with anti-hDEC-OVA (Fig. 13A, left) and showed similar intrinsic antigen presenting activity when pulsed with peptides (Fig. 13C, left). When antigen was titrated under conditions where DCs were present in excess, the results of *in vitro* targeting mirrored those obtained *in vivo* in that the two subsets were equivalent for MHCI cross-presentation and also that $CD8^-$ DCs were intrinsically more efficient than $CD8^+$ DCs in processing and presenting antigens in MHCII (Fig. 13B, left). Small differences in presentation were found when the number of antigen presenting cells were titrated: $CD8^+$ DCs were about 3 fold more effective than $CD8^-$ DCs for cross-presentation when less than 10,000 DCs were present (Fig. 14). In contrast, $CD8^-$ DCs were several orders of magnitude more efficient than $CD8^+$ DCs for MHCII presentation, irrespective on the number of DCs assayed (Fig. 14). In conclusion, $CD8^-$ and $CD8^+$ splenic DCs are similar in terms of their ability to cross-present antigens on MHCI when the antigen is captured by DEC-205.

Figure 13. Antigen presentation after receptor-mediated endocytosis *in vitro*. A-C, APCs from CD11c-hDEC mice were isolated from naïve (solid lines) or mBSA-CFA immunized (dashed lines) mice (left panels), or cultured from bone marrow (right panels). A, The Y-axis shows relative cell-associated OVA, as measured by rabbit anti-OVA and developed with anti-rabbit-HRP, after targeting with the indicated concentrations of anti-hDEC-OVA on the X-axis. B, Activation and proliferation of OTI T cells (upper panels) and OTII T cells (lower panels) in response to OVA containing APCs. The Y-axis shows the percentage of divided T cells. C, Graphs show OTI (upper panels) and OTII (lower panels) proliferation in response to peptide pulsed APCs. D-F, APCs were isolated from spleen or cultured from bone marrow of WT mice. Dashed lines indicate LPS activation. D and E, as in A and B, but targeting was with anti-mouse-DEC-OVA. F, As in C. Graphs represent pooled data from 3-7 independent experiments.

Figure 13

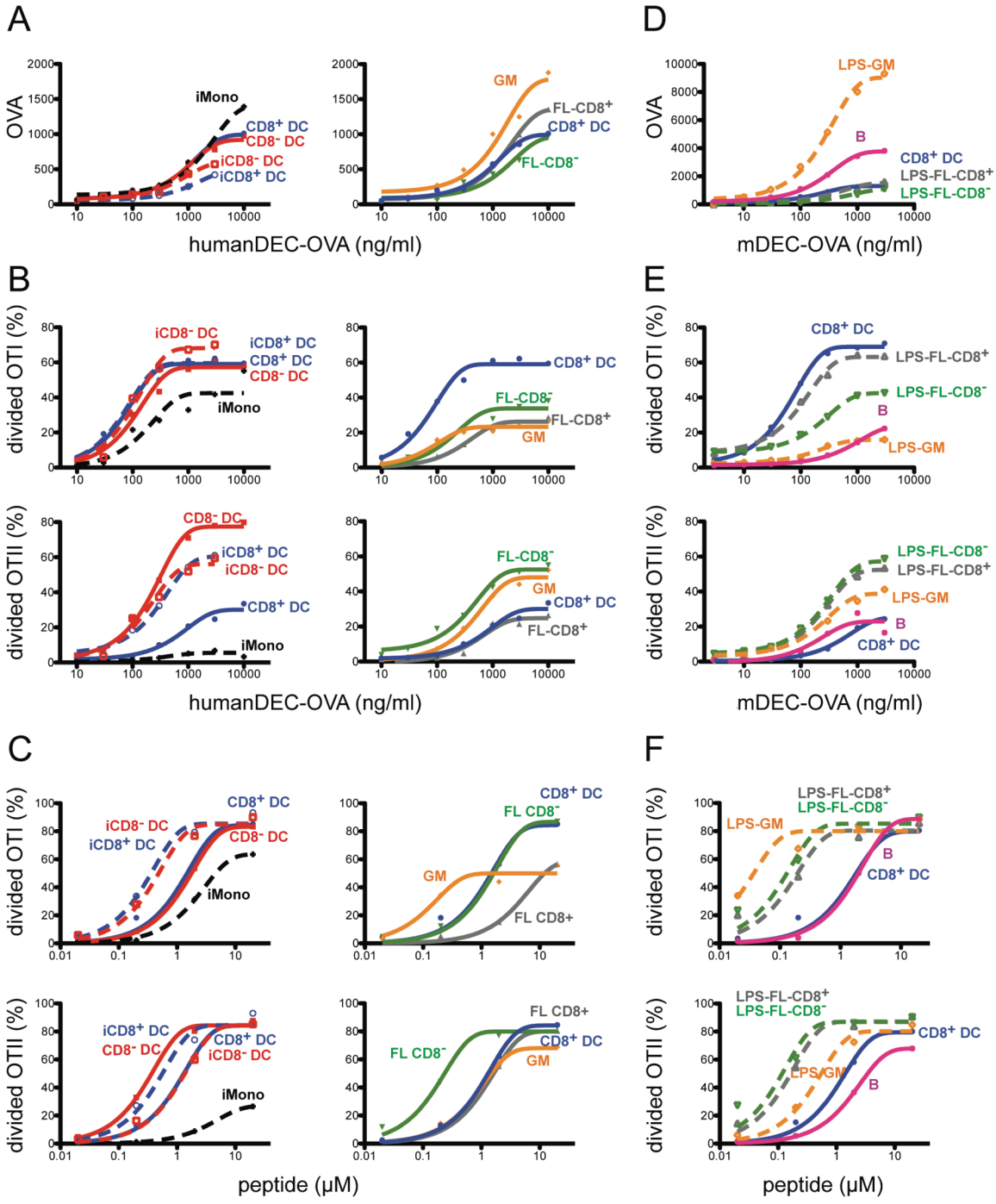
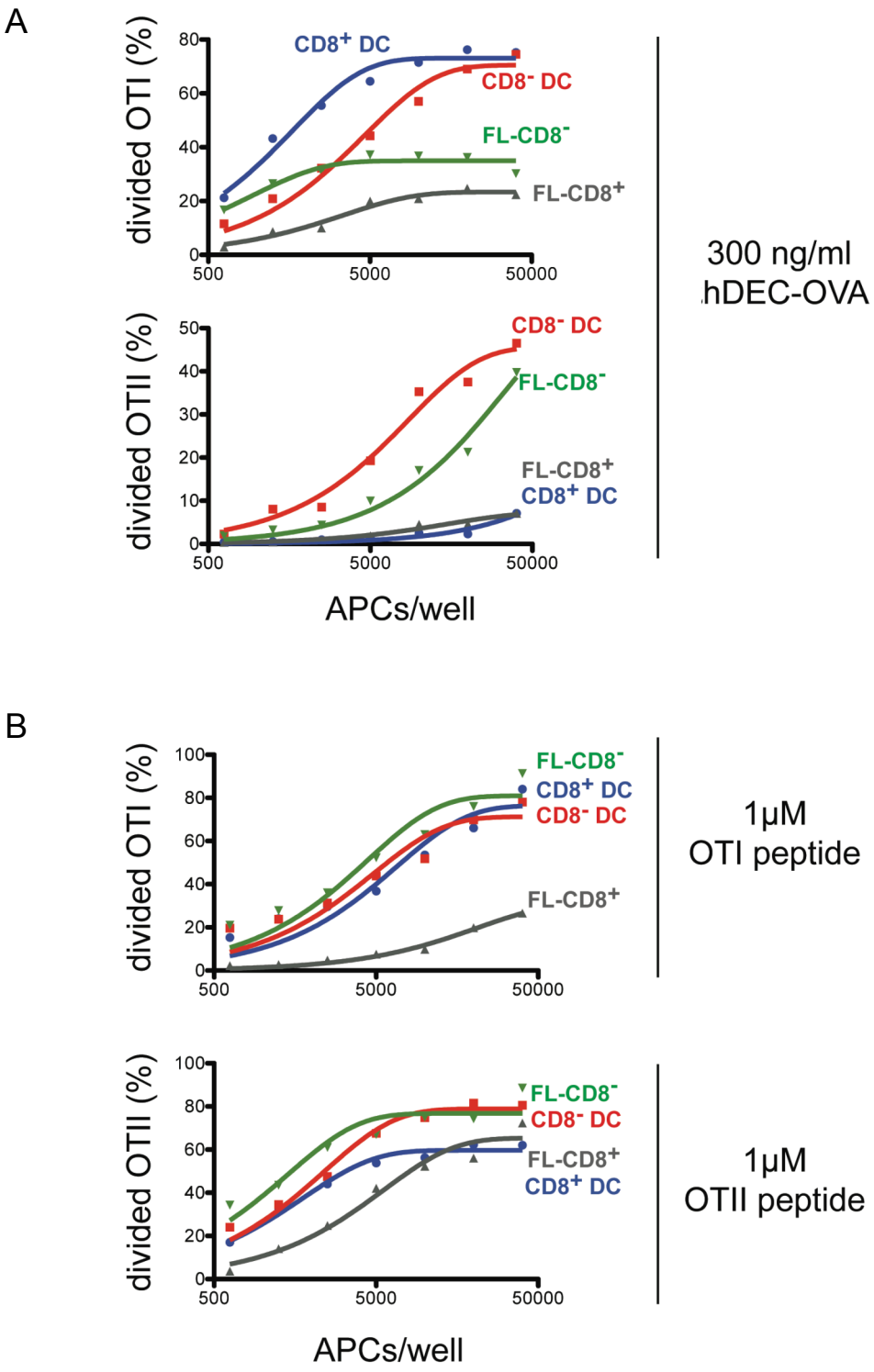

A
OVA
iMono
CD8+ DC
CD8- DC
iCD8- DC
iCD8+ DC
GM
FL-CD8+
CD8+ DC
FL-CD8-
humanDEC-OVA (ng/ml)
D
OVA
LPS-GM
B
CD8+ DC
LPS-FL-CD8+
LPS-FL-CD8-
mDEC-OVA (ng/ml)
B
divided OTI (%)
iCD8- DC
iCD8+ DC
CD8+ DC
CD8- DC
iMono
CD8+ DC
FL-CD8-
FL-CD8+
GM
divided OTII (%)
CD8- DC
iCD8+ DC
iCD8- DC
CD8+ DC
iMono
FL-CD8-
GM
CD8+ DC
FL-CD8+
humanDEC-OVA (ng/ml)
E
divided OTI (%)
CD8+ DC
LPS-FL-CD8+
LPS-FL-CD8-
B
LPS-GM
divided OTII (%)
LPS-FL-CD8-
LPS-FL-CD8+
LPS-GM
B
CD8+ DC
mDEC-OVA (ng/ml)
C
divided OTI (%)
CD8+ DC
iCD8- DC
iCD8+ DC
CD8- DC
iMono
CD8+ DC
FL CD8-
GM
FL CD8+
divided OTII (%)
iCD8+ DC
CD8- DC
CD8+ DC
iCD8- DC
iMono
FL CD8+
FL CD8-
CD8+ DC
GM
peptide (μM)
F
divided OTI (%)
LPS-FL-CD8+
LPS-FL-CD8-
LPS-GM
B
CD8+ DC
divided OTII (%)
LPS-FL-CD8+
LPS-FL-CD8-
CD8+ DC
LPS-GM
B
peptide (μM)

Figure 14. Antigen presentation after receptor-mediated endocytosis *in vitro:* APC titration. APCs were isolated from spleen or cultured from bone marrow of naïve CD11c-hDEC mice. A, Activation and proliferation of OTI T cells (upper panel) and OTII T cells (lower panel) in response to APCs targeted with 300 ng/ml of anti-hDEC-OVA. The Y-axis shows the percentage of divided T cells. The X-axis shows the number of APCs plated in each 96-well. B, Graphs show OTI (upper panel) and OTII (lower panel) proliferation in response to peptide pulsed APCs. Graphs represent pooled data from 2 independent experiments.

Figure 14


A
divided OTI (%)
CD8+ DC
CD8- DC
FL-CD8-
FL-CD8+
divided OTII (%)
CD8- DC
FL-CD8-
FL-CD8+
CD8+ DC
APCs/well
300 ng/ml
hDEC-OVA
B
divided OTI (%)
FL-CD8-
CD8+ DC
CD8- DC
FL-CD8+
1μM
OTI peptide
divided OTII (%)
FL-CD8-
CD8- DC
FL-CD8+
CD8+ DC
APCs/well
1μM
OTII peptide

During some infections or inflammation, monocytes become activated, acquire some DC features and can differentiate into tip-DCs (Naik et al., 2006; Serbina et al., 2008; Serbina et al., 2003). At least in some situations, this process is GM-CSF dependent (Cook et al., 2004) (Fig. 15). Conversely, conventional DCs are FL-dependent, GM-CSF-independent and derived from pre-DCs (Waskow et al., 2008). In order to compare activated monocytes to conventional DCs, hDEC-205 transgenic mice were immunized with antigen in complete Freund's adjuvant (CFA) and then challenged with antigen to induce monocyte activation (Cook et al., 2004). Activated monocytes were $CD11c^+$ but also $Ly6C^+$, and had similar levels of CD86, 2- fold higher levels of MHCI but lower levels of MHCII when compared to conventional DCs from naïve mice (Fig. 15 and 16). Activated monocytes, unlike DCs, were positive for M-CSF receptor but negative for Flk2 (FL receptor), DEC-205 and DCIR2 (Fig. 15). Conventional DCs isolated from the immunized mice ($iCD8^+$ and $iCD8^-$ DCs) had higher levels of CD86, MHCI and MHCII when compared to control DCs from naïve mice (Fig. 15 and 16).

Activated monocytes and conventional DCs were purified by cell sorting, as described in methods, and compared for antigen presentation after targeting with anti-hDEC-OVA. All cell types isolated from immunized mice were similar to control DCs in antigen uptake after anti-hDEC-OVA targeting (Fig. 13A, left) (Platt et al., 2010). However, DCs from immunized mice were more efficient than their naïve counterparts with regards to the presentation of pulsed peptides. On the other hand, activated monocytes were less efficient than DCs, especially for MHCII presentation, when pulsed with peptides (Fig. 13C, left). Consistent with the peptide presentation experiments, activated monocytes

Figure 15. Activated monocytes differ from conventional DCs in that they are GM-CSF dependent and do not express Flk2 but express M-CSF receptor. WT and GMCSF-Rβ KO (GMR KO) mice were immunized with mBSA-CFA and i.p. challenged with mBSA 24 hours before analysis. A, Representative flow cytometry profile of splenocytes (after exclusion of T cells, B cells, NK cells, granulocytes and pDCs) showing CD11c and CD11b expression (upper panels). Sorting strategy for activated monocytes ($CD11c^+$) and $CD8^-$DCs, after further gating on $CD11b^+$ $CD8^-$ cells (lower panels). B, Absolute number of different cell populations in the spleen, where N stands for naïve mice and I for immunized mice. Graph shows mean ± SE, of two independent experiments with three mice/group each. C, Representative histograms show CD86, MHCI and MHCII expression on the different cell populations. D, Representative dot-plots show expression of CD11b, CD11c, DCIR2, DEC-205, CD135 (Flk2) and CD115 (M-CSF receptor) on monocytes, $CD8^+$ DCs, $CD8^-$ DCs and activated monocytes ($CD11c^+$, iMono).

Figure 15

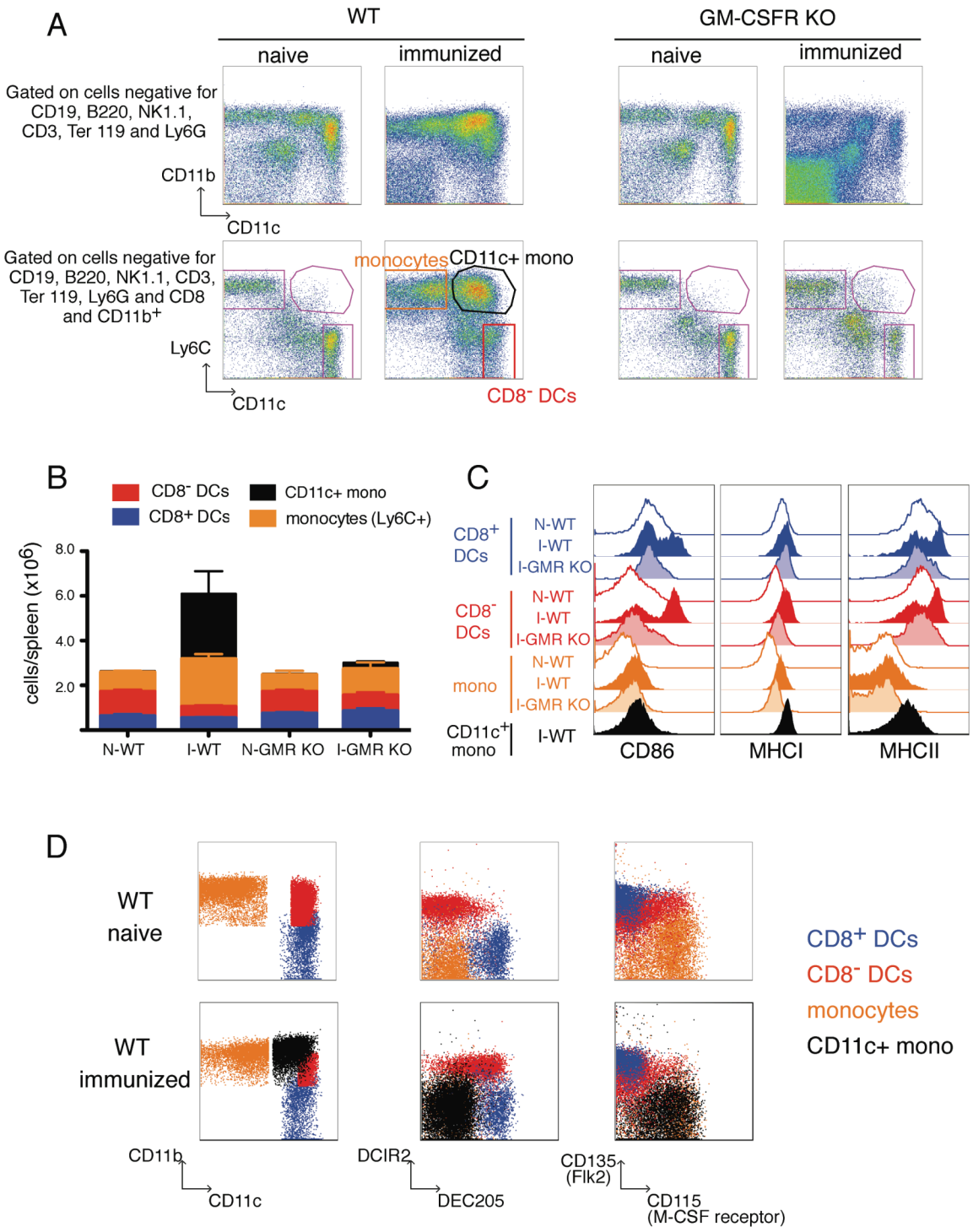
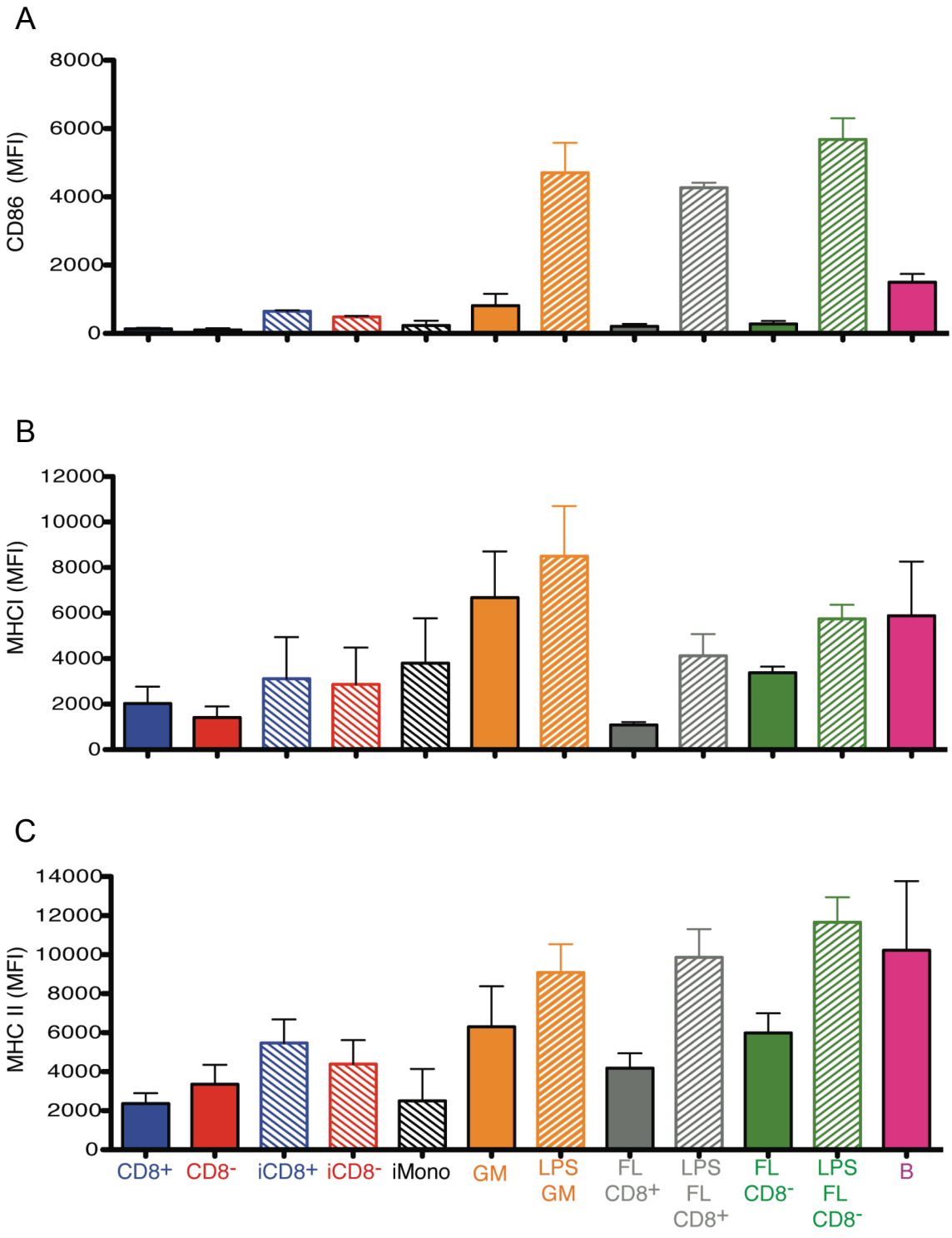

A
WT
GM-CSFR KO
naive
immunized
naive
immunized
Gated on cells negative for CD19, B220, NK1.1, CD3, Ter 119 and Ly6G
CD11b
CD11c
Gated on cells negative for CD19, B220, NK1.1, CD3, Ter 119, Ly6G and CD8 and CD11b+
monocytes
CD11c+ mono
Ly6C
CD11c
CD8- DCs
B
CD8- DCs
CD8+ DCs
CD11c+ mono
monocytes (Ly6C+)
cells/spleen (x10^6)
8.0
6.0
4.0
2.0
N-WT
I-WT
N-GMR KO
I-GMR KO
C
CD8+ DCs
CD8- DCs
mono
CD11c+ mono
N-WT
I-WT
I-GMR KO
I-WT
CD86
MHCI
MHCII
D
WT naive
WT immunized
CD11b
CD11c
DCIR2
DEC205
CD135 (Flk2)
CD115 (M-CSF receptor)
CD8+ DCs
CD8- DCs
monocytes
CD11c+ mono

Figure 16. CD86, MHCI and MHCII expression. CD86 (A), MHCI (B) and MHCII (C) expression on the indicated APCs. The mean fluorescence intensity is represented in the Y-axis. Data represents the mean± SE from 3 independent experiments.

Figure 16


A
CD86 (MFI)
8000
6000
4000
2000
0
B
MHCI (MFI)
12000
10000
8000
6000
4000
2000
0
C
MHC II (MFI)
14000
12000
10000
8000
6000
4000
2000
0
CD8+
CD8-
iCD8+
iCD8-
iMono
GM
LPS GM
FL CD8+
LPS FL CD8+
FL CD8-
LPS FL CD8-
B

performed poorly in MHCII presentation but showed levels of cross-presentation that approached those of conventional DCs (Fig. 13B, left). Thus, activated monocytes resemble conventional DCs in their ability to cross-present antigens captured by receptor-mediated endocytosis. However, under the same conditions, activated monocytes are far inferior to naïve or activated conventional spleen DCs with regards to MHCII presentation.

DCs can be obtained by culturing murine bone marrow cells with GM-CSF (GM-DCs) (Inaba et al., 1992a) or FL (FL-DCs)(Brasel et al., 2000). FL-DCs are heterogeneous and contain cells that resemble $CD8^+$ and $CD8^-$ splenic DCs (FL-$CD8^+$ and FL-$CD8^-$ DCs respectively) (Naik et al., 2005; Weigel et al., 2002). Both FL- and GM-DCs can be activated with TLR agonists, such as LPS, to increase the expression of MHC and co-stimulatory molecules (Brasel et al., 2000; Cella et al., 1997; Pierre et al., 1997) as well as antigen processing (Inaba et al., 2000; Trombetta et al., 2003) and the stability of MHC-peptide complexes on the cell surface (Shin et al., 2006; Trombetta and Mellman, 2005). Moreover, endogenous mouse DEC-205 expression is induced on cultured DCs upon TLR ligation (Brasel et al., 2000; Inaba et al., 1995) and also on B cells after culture with LPS and IL-4 (Fig. 10).

In order to compare the ability of tissue culture-derived DCs and conventional DCs from spleen to present antigens captured by receptor-mediated endocytosis, GM- and FL-DCs obtained from hDEC transgenic mice were incubated with anti-hDEC-OVA. CD86, MHCI and MHCII expression by GM- and FL-DCs was equal to or higher than spleen

DCs (Fig. 16). But GM-DCs accumulated more OVA than spleen DCs or FL-DCs after anti-hDEC-OVA targeting (Fig. 13A, right). Nevertheless, GM-DCs were less effective than spleen DCs in cross-presentation (Fig. 13B, right). Consistent with their high levels of MHCII expression, GM-DCs were efficient in presenting pulsed peptides to MHCII restricted T cells (Fig. 13C, right). However, the same cells were intermediate, between $CD8^+$ and $CD8^-$ DCs, for MHCII presentation after antigen capture by receptor-mediated endocytosis (Fig. 13B, right).

FL-DCs were separated into $CD8^-$ ($SIRP\alpha^{hi}$) and $CD8^+$ ($CD24^{hi}$) subsets. Both FL-DC subsets induced less OTI proliferation than splenic DCs (Fig. 13 B, right). But FL-$CD8^+$ DCs were also almost 10-fold less efficient than their splenic counterparts for MHCI presentation after exogenous peptide loading (Fig. 13 C, right). For MHCII presentation FL-$CD8^-$ DCs were more effective that FL-$CD8^+$ DCs, the latter showing similar efficiency as their splenic counterpart $CD8^+$ DCs (Fig. 13 B, right). Thus, both GM- and FL-DCs are capable of MHCI and MHCII presentation when antigen is captured by receptor-mediated endocytosis; however, these cells are less active than conventional spleen DCs.

In order to determine how activation by TLR ligation alters DCs ability to present antigens acquired through receptor-mediated endocytosis, GM- or FL-DCs were stimulated with LPS and antigen presentation was assessed after targeting with anti-mouse-DEC-OVA. Naïve splenic $CD8^+$ DCs and LPS/IL-4 activated B cells were used as controls. As expected, stimulation with LPS enhanced expression of co-stimulatory

molecules, MHCI and MHCII in all culture-derived DCs (Fig. 16) (Trombetta and Mellman, 2005). GM-DCs activated with LPS expressed high levels of DEC-205 and thus captured 5-fold more antigen than any of the other DCs tested (Fig. 13D). In addition, when loaded with exogenous peptide, LPS-GM-DCs performed better than control $CD8^+$ DCs in activating MHCI and MHCII-dependent T cell responses (Fig. 13F). But, LPS-GM-DCs were far less effective than $CD8^+$ DCs and no better than resting GM-DCs in cross-presentation of antigens acquired by receptor-mediated endocytosis (Fig. 13E). Finally, LPS stimulation also decreased the relative efficiency of GM-DCs with regards to processing antigen to be presented on MHCII (Fig. 13E).

LPS-FL-DCs captured similar amounts of antigen as did control splenic $CD8^+$ DCs (Fig. 13D) but showed increased activation of MHCI and MHCII restricted T cells after peptide loading (Fig. 13F). Surprisingly, LPS stimulation had a positive impact on antigen presentation on FL-$CD8^+$ but not on FL-$CD8^-$ DCs (Fig. 13D and E). In conclusion, when antigens were captured by receptor-mediated endocytosis, LPS failed to enhance MHCI and MHCII presentation by all cultured DCs, with the exception of FL-$CD8^+$ DCs.

LPS/IL-4 activated B cells, which were used as an additional control, expressed higher levels of CD86, MHCI and MHCII than splenic DCs (Fig. 16), captured more antigen than $CD8^+$ DCs (Fig. 13D) and were equivalent to $CD8^+$ DCs in presenting exogenously loaded peptide to MHCI and MHCII restricted T cells (Fig. 13F). Consistent with these observations, activated B cells were also similar to $CD8^+$ DCs in antigen presentation to

MHCII restricted T cells after antigen targeting to DEC-205 (Fig. 13E). On the other hand, B cell blasts induced far less OTI proliferation and were much less efficient than DCs in cross-presentation (Fig. 13E).

In conclusion, when antigen is captured by receptor-mediated endocytosis, FL-DCs (both $CD8^+$ and $CD8^-$) resemble conventional spleen DCs with regards to efficiency of antigen presentation. Furthermore, antigen delivery by endocytosis through DEC-205 is not sufficient to ensure highly efficient cross-presentation, since B cells and LPS-GM-DCs were almost 10-fold less effective in cross-presentation than DCs.

To determine whether the observed differences in antigen presentation were cell intrinsic or dependent on the route of antigen capture, cells were loaded with antigen by pinocytosis, using high concentrations of soluble OVA. In those experiments, OVA-biotin was used to facilitate the measurement of antigen uptake. Although some preparations of OVA display mannose residues that serve as ligands for mannose receptors, there was no detectable binding of OVA to any of the cell types tested.

Both splenic DC subsets obtained from naïve and immunized mice showed similar levels of OVA endocytosis (Fig. 17A, left). Despite their similarities in antigen uptake by endocytosis and presentation of exogenous peptides, OVA captured by bulk pinocytosis was presented far more effectively by $CD8^-$ than $CD8^+$ DCs to MHCII restricted T cells (Fig. 17B and C, left). In addition, DCs obtained from immunized mice were more efficient at MHCII antigen presentation than naïve DCs, but the differences between the

subsets did not change qualitatively (Fig. 17B, left). Therefore, $CD8^-$ DCs are more efficient than $CD8^+$ DCs at presentation to MHCII restricted T cells, irrespective of the route of antigen uptake or activation status.

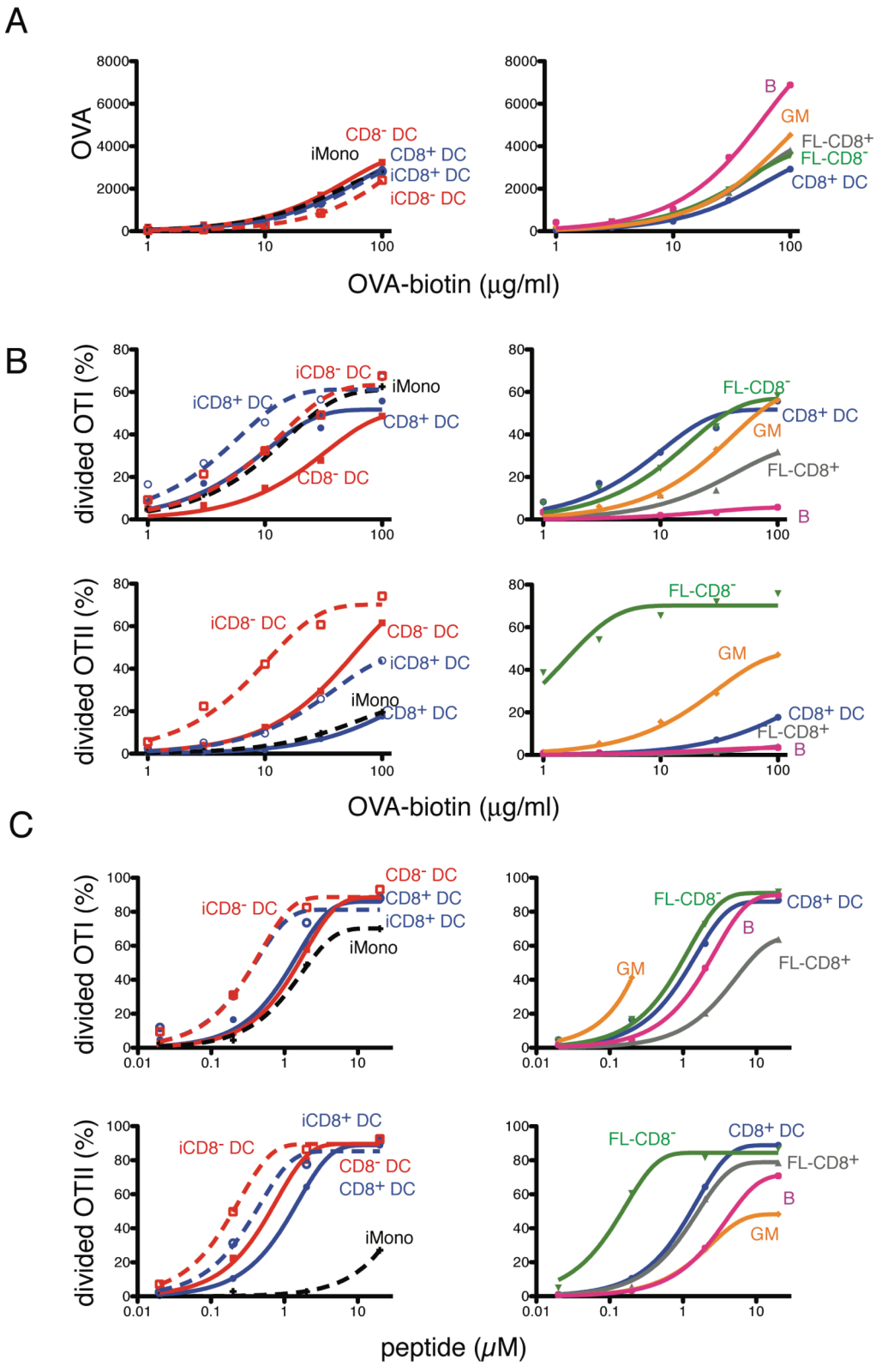
Despite antigen uptake at levels similar to conventional DCs, activated monocytes showed low levels of MHCII presentation of pinocytosed antigen, which correlated with low levels of presentation with pulsed peptide (Fig. 17, left). Yet, activated monocytes were similar to spleen DCs for cross-presentation (Fig. 17B, left). Thus activated monocytes can cross-present pinocytosed antigens as efficiently as spleen DCs; nevertheless, these cells are far less effective in MHCII presentation.

Among cultured DCs, FL-DCs acquired similar amounts of OVA by pinocytosis as their spleen DC counterparts (Fig. 17A, right). Whereas FL-$CD8^-$ DCs were equivalent to spleen DCs for cross-presentation and more efficient with regards to MHCII presentation of pinocytized antigen, FL-$CD8^+$ DCs were less effective in both tasks (Fig. 17B, right). GM-DCs accumulated similar amounts of OVA by bulk phase pinocytosis and performed similarly to $CD8^+$ DCs for cross-presentation and intermediate between the two DC subsets for MHCII presentation (Fig. 17A and B, right).

In contrast, activated B cells, which were active in endocytosis and presentation of peptides, were nearly inactive in processing antigen acquired by bulk phase pinocytosis for presentation on MHCII or MHCI (Fig. 17, right). Therefore, both the cell type and the route of endocytosis influence the efficiency of antigen presentation.

Figure 17. Antigen presentation after pinocytosis *in vitro*. APCs were isolated from spleen or derived from bone marrow of WT mice. (A) The Y-axis shows relative cell-associated OVA, developed with streptavidin-HRP, after incubation of the APCs with the indicated concentrations of OVA-biotin on the X-axis. (B) Activation and proliferation of OTI T cells (upper panels) and OTII T cells (lower panels) in response to OVA containing APCs. The Y-axis shows percentage of divided T cells. (C) Graphs show OTI (upper panels) and OTII (lower panels) proliferation in response to peptide pulsed APCs. Graphs represent pooled data from 3-5 independent experiments.

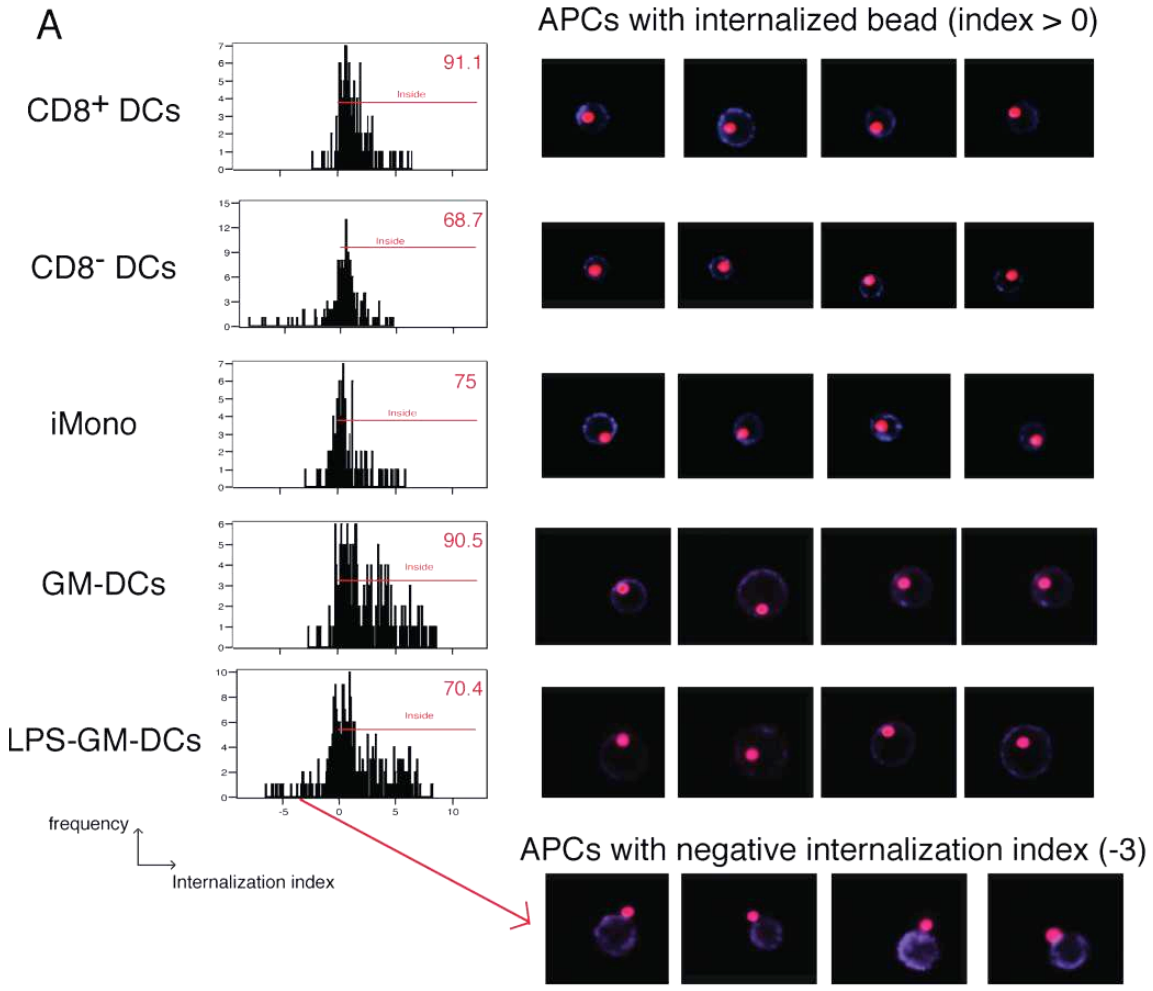
Figure 17

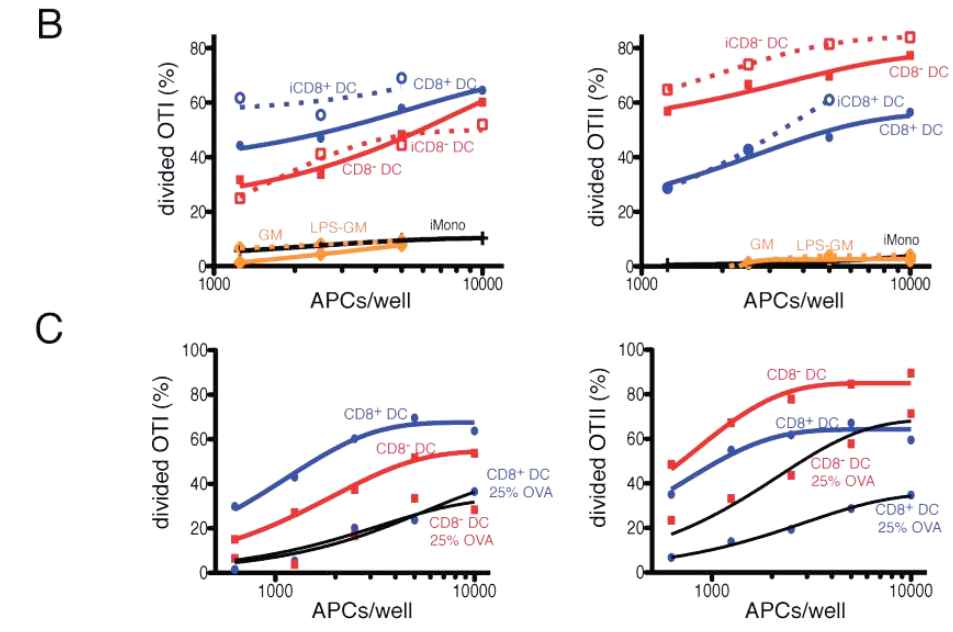

A
OVA
8000
6000
4000
2000
0
1
10
100
CD8⁻ DC
iMono
CD8⁺ DC
iCD8⁺ DC
iCD8⁻ DC
B
GM
FL-CD8⁺
FL-CD8⁻
CD8⁺ DC
OVA-biotin (μg/ml)
B
divided OTI (%)
iCD8⁻ DC
iCD8⁺ DC
iMono
CD8⁺ DC
CD8⁻ DC
FL-CD8⁻
CD8⁺ DC
GM
FL-CD8⁺
B
divided OTII (%)
iCD8⁻ DC
CD8⁻ DC
iCD8⁺ DC
iMono
CD8⁺ DC
FL-CD8⁻
GM
CD8⁺ DC
FL-CD8⁺
B
OVA-biotin (μg/ml)
C
divided OTI (%)
CD8⁻ DC
iCD8⁻ DC
CD8⁺ DC
iCD8⁺ DC
iMono
FL-CD8⁻
CD8⁺ DC
B
GM
FL-CD8⁺
divided OTII (%)
iCD8⁺ DC
iCD8⁻ DC
CD8⁻ DC
CD8⁺ DC
iMono
FL-CD8⁻
CD8⁺ DC
FL-CD8⁺
B
GM
peptide (μM)

To further analyze the effects of the route of antigen uptake on antigen presentation, OVA was delivered to antigen-presenting cells by phagocytosis of OVA-adsorbed polystyrene beads. Flow cytometry was used to purify cells that had captured a single fluorescent bead to normalize the amount of antigen captured. Internalization was confirmed by analysis on Image Stream cytometry (Fig. 18A), which combines flow cytometry with imaging of individual cells. Naïve and activated splenic DCs presented antigen acquired by phagocytosis in a manner that was similar to antigen captured by receptor-mediated or bulk phase pinocytosis (Fig. 18B). $CD8^-$ DCs remained more efficient than $CD8^+$ in MHCII presentation, and cross-presentation was similar in both types of DCs (Fig. 18B and C). Decreasing the amount of OVA on the beads did not alter the results (Fig. 18C). However, neither activated monocytes nor GM-DCs, nor LPS activated GM-DCs presented phagocytized antigens to MHCI or MHCII restricted T cells to any appreciable degree (Fig. 18B). Similar results were obtained when splenic monocytes from *Listeria* infected mice were assayed (tip-DCs) (Fig. 19). In conclusion, activated monocytes and GM-DCs are far less effective in antigen presentation than conventional DCs when the antigen is acquired by phagocytosis.

Figure 18. Antigen presentation after phagocytosis. APCs were isolated from spleen of naïve or mBSA-CFA immunized mice. GM-DCs were derived from bone marrow. Enriched populations were incubated with OVA-beads before sorting. APCs that had captured one OVA-bead were sorted. A, After sorting, cells were stained on ice with CD11c-, CD11b-, CD8- and MHCII-biotin, followed by streptavidin Pacific blue and analyzed on Image Stream 100 (Amnis). Histograms show distribution of internalization scores for the different APC populations, where positive scores indicate internalization. The percentage of cells with internalized beads is shown in each histogram and 4 representative cell images are shown on the right. Representative images from cells that had a negative internalization score are also shown (lower images). B, Activation and proliferation of OTI T cells (left panel) and OTII T cells (right panel) after incubation with the number of APCs indicated in the X-axis, which were sorted to contain a single OVA-bead. The Y-axis shows percentage of divided T cells. C, as in B, but DCs from naïve mice were also incubated with beads adsorbed with 25% OVA and 75% KLH protein. B and C represent pooled data from 2-4 independent experiments.

Figure 18

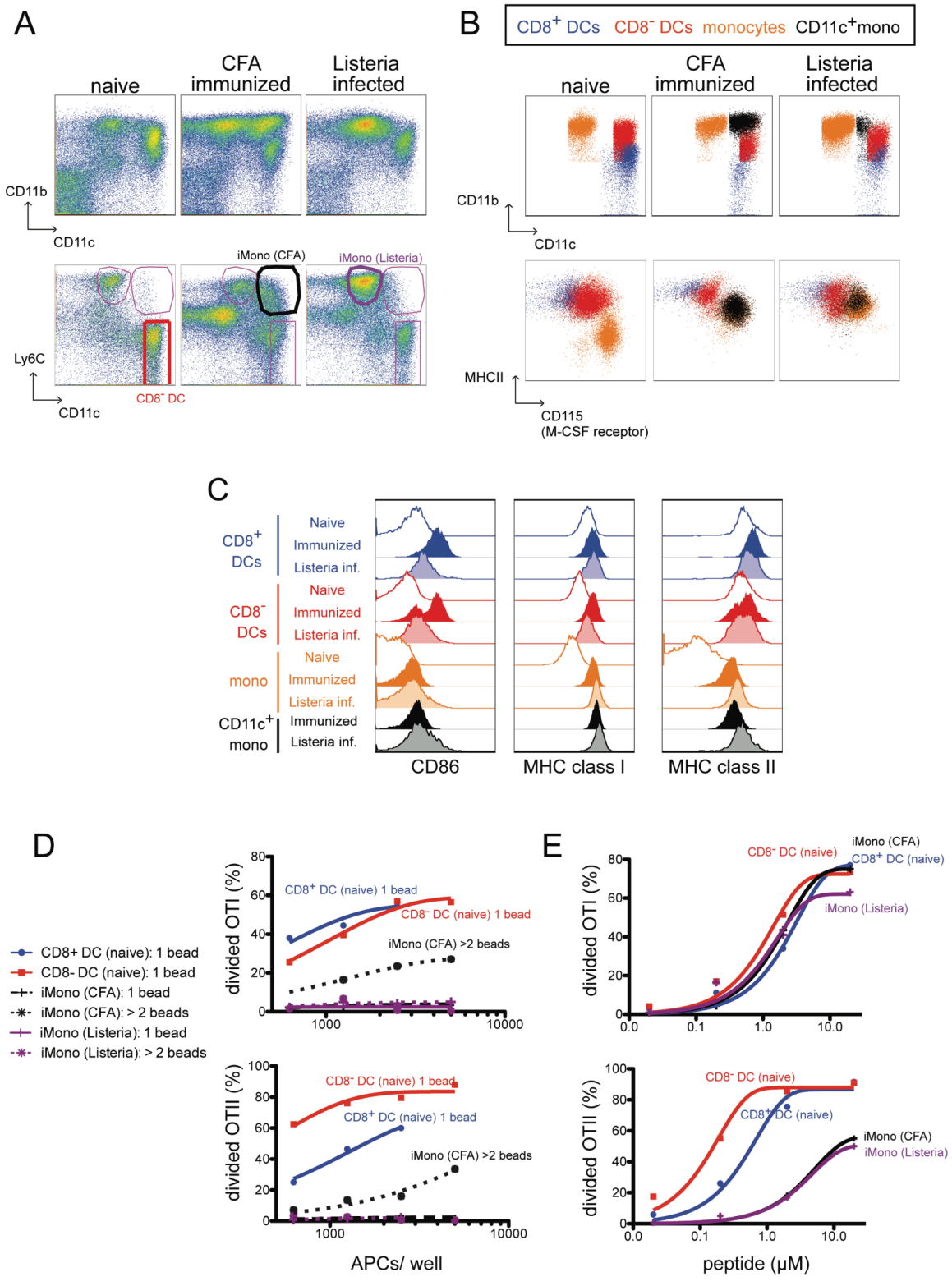

A
APCs with internalized bead (index > 0)
CD8+ DCs
91.1
Inside
CD8- DCs
68.7
Inside
iMono
75
Inside
GM-DCs
90.5
Inside
LPS-GM-DCs
70.4
Inside
frequency
Internalization index
APCs with negative internalization index (-3)


B
divided OTI (%)
iCD8+ DC
CD8+ DC
iCD8- DC
CD8- DC
GM
LPS-GM
iMono
APCs/well
divided OTII (%)
iCD8- DC
CD8- DC
iCD8+ DC
CD8+ DC
GM
LPS-GM
iMono
APCs/well

C
divided OTI (%)
CD8+ DC
CD8- DC
CD8+ DC 25% OVA
CD8- DC 25% OVA
APCs/well
divided OTII (%)
CD8- DC
CD8+ DC
CD8- DC 25% OVA
CD8+ DC 25% OVA
APCs/well

Figure 19. Comparison of tip-DCs elicited by *Listeria monocytogenes* infection and activated monocytes elicited by mBSA-CFA immunization. WT mice were immunized with mBSA-CFA and i.p. challenged with mBSA 24 hours before analysis. Alternatively, WT mice were infected with 3,000 *Listeria monocytogenes* i.v. and analyzed 48 hrs later. A, Representative flow cytometry profile of splenocytes (after exclusion of T cells, B cells, NK cells, granulocytes and pDCs) showing CD11c and CD11b expression (upper panels). Sorting scheme (lower panels), after further gating on $CD11b^+$ $CD8^-$ cells, for $CD8^-$ DCs, activated monocytes ($CD11c^+$ monocytes from immunized mice; iMono CFA), or tip-DCs (monocytes from Listeria infected mice; iMono Listeria). B, Representative dot-plots show expression of CD11b, CD11c, CD115 (M-CSF receptor) and MHCII on $CD8^+$ DCs, $CD8^-$ DCs, monocytes and $CD11c^+$ monocytes. C, Representative histograms show CD86, MHCI and MHCII expression on the indicated cell populations. D, DCs ($CD8^+$ and $CD8^-$) were isolated from spleen of naïve mice and iMono from mBSA-CFA immunized mice ($CD11c^+$ monocytes) or Listeria infected mice. Enriched populations were incubated with OVA-beads before sorting. Activation and proliferation of OTI T cells (top panel) and OTII T cells (lower panel) after incubation with the number of APCs indicated in the X-axis, which were sorted to contain a single OVA-bead or more than 2 beads, as indicated. The Y-axis shows percentage of divided T cells. E, as in D, but cells that did not capture any beads were sorted. $5x10^3$ APCs were pulsed with the indicated concentrations of peptides in the X-axis and subsequently co-cultured with OTI (top panel) or OTII (lower panel) T cells. The Y-axis shows percentage of divided T cells.

Figure 19


A
naive
CFA immunized
Listeria infected
CD11b
CD11c
iMono (CFA)
iMono (Listeria)
Ly6C
CD8⁻ DC
CD11c
B
CD8⁺ DCs
CD8⁻ DCs
monocytes
CD11c⁺mono
naive
CFA immunized
Listeria infected
CD11b
CD11c
MHCII
CD115 (M-CSF receptor)
C
CD8⁺ DCs
Naive
Immunized
Listeria inf.
CD8⁻ DCs
Naive
Immunized
Listeria inf.
mono
Naive
Immunized
Listeria inf.
CD11c⁺ mono
Immunized
Listeria inf.
CD86
MHC class I
MHC class II
D
CD8+ DC (naive): 1 bead
CD8- DC (naive): 1 bead
iMono (CFA): 1 bead
iMono (CFA): > 2 beads
iMono (Listeria): 1 bead
iMono (Listeria): > 2 beads
divided OTI (%)
CD8⁺ DC (naive) 1 bead
CD8⁻ DC (naive) 1 bead
iMono (CFA) >2 beads
1000
10000
divided OTII (%)
CD8⁻ DC (naive) 1 bead
CD8⁺ DC (naive) 1 bead
iMono (CFA) >2 beads
1000
10000
APCs/ well
E
divided OTI (%)
CD8⁻ DC (naive)
iMono (CFA)
CD8⁺ DC (naive)
iMono (Listeria)
0.0
0.1
1.0
10.0
divided OTII (%)
CD8⁻ DC (naive)
CD8⁺ DC (naive)
iMono (CFA)
iMono (Listeria)
0.0
0.1
1.0
10.0
peptide (μM)

Chapter 6

Discussion

Ralph M. Steinman and Zanvil A. Cohn first described dendritic cells in 1973 (Steinman and Cohn, 1973), and the new cells were characterized by their morphology and excellence at inducing mixed leukocyte reactions (Steinman and Witmer, 1978). It took many years and compelling evidence to convince the scientific community that DCs were distinct from macrophages. The main function of DCs is to present antigen to T cells, controlling both tolerance and immunity. However, there is still no single morphologic feature, function or unique expressed protein that can unambiguously identify DCs. For example, CD11c expression is key for identifying murine DCs, but it is also abundant in alveolar macrophages.

Identifying unique genes required for DC development or for specifying DC functions is crucial. Using gene expression data from DCs, DC-progenitors and monocytes, our lab has identified candidate genes that may provide unique markers for the DC lineage, conserved in mice and humans. This information is necessary to resolve the monocyte, macrophage and DC definition controversy that still persists (Geissmann et al., 2010) and will help in settling the classification of myeloid cells.

Elegant work has shown that DCs derive from unique progenitors that are distinct from monocytes and macrophages (Liu et al., 2009; Naik et al., 2007; Onai et al., 2007).

Therefore a DC identity based on ontogeny can be defined, at least in the steady state. During inflammation, monocytes differentiate into cells that have several features of DCs (CD11c and MHCII expression) (Serbina et al., 2008), but their role in eliciting immunity by antigen presentation is not well defined. Antigen presentation to T cells is the main function attributed to DCs, while monocytes and macrophages have a prominent role in the inflammation process and catabolism.

In this thesis I explore antigen presentation by DCs and other antigen presenting cells. I find that although other antigen presenting cells, such as activated monocytes can present antigen, they are less efficient than DCs. Also, the route of antigen uptake has a profound impact on their ability to process antigen for MHC presentation. DCs derived from pre-DCs present antigens irrespective of the route of antigen capture. Importantly, I also find differences for MHCII presentation between DC subsets. Thus I identify both unifying functional features for DCs and a division of labor among different subsets, based on antigen presentation.

Early in the history of DCs it was observed that different subsets co-existed in lymphoid organs (Crowley et al., 1989). However, many of the molecules differentially expressed by DC subsets do not appear to confer a specialized function. Comparison of DCs from WT mice and mice deficient for CD8α (Kronin et al., 1997) or DEC-205 (Iyoda, 2002; data not shown) - two proteins widely used to define DC subsets - failed to show these molecules playing a role in DC development or T cell activation. Yet the advent of new technologies and further characterization of DC subsets, comparing global gene

expression (Dudziak et al., 2007; Edwards et al., 2003a; Robbins et al., 2008), definitively showed that distinct subsets are related but unique. For example $CD8^+$ and $CD8^-$ DCs differ in expression of C-type lectins (Dudziak et al., 2007) and TLRs (Edwards et al., 2003b). Thus each DC subset displays unique abilities to bind and respond to different pathogens.

Here I have addressed differences between splenic $CD8^+$ and $CD8^-$ DCs in antigen processing and presentation. Previous studies have shown that cell-associated antigens are cross-presented by $CD8^+$ DCs (den Haan et al., 2000; Hildner et al., 2008; Schulz and Reis e Sousa, 2002), which can be attributed to their higher ability to capture dead cells (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002). Both $CD8^+$ and $CD8^-$ DCs cross-present antigens expressed by bacteria (Schulz and Reis e Sousa, 2002; Yrlid and Wick, 2002). When soluble antigen is administrated to mice, it has been shown that $CD8^+$ DCs are better at cross-presentation, while $CD8^-$ DCs are better at MHCII presentation (Pooley et al., 2001; Schnorrer et al., 2006). However, a careful analysis of antigen uptake shows that $CD8^+$ DCs may actually capture more soluble antigen than $CD8^-$ DCs (Idoyaga et al., 2009; Pooley et al., 2001; Schnorrer et al., 2006). Likewise, many reports failed to ensure that both DC subsets had equal access to antigen (Belz et al., 2005) or used the same mechanism for antigen internalization, precluding conclusions on the antigen presentation efficiency of DC subsets.

The advent of recombinant antibodies carrying antigen that bind to DC receptors enabled comparison between different DC subsets and receptors. Transgenic mice expressing

human DEC-205 molecules driven by the CD11c promoter, provided expression of a shared receptor on distinct DC subsets. In addition, previous work with anti-DEC antigen targeting validated this antigen delivery strategy for MHC presentation, without affecting DC maturation (Bonifaz et al., 2002; Bonifaz et al., 2004; Hawiger et al., 2001; Hawiger et al., 2004).

Using anti-hDEC chimeric antibodies carrying antigen to both $CD8^+$ and $CD8^-$ splenic DCs, I show that $CD8^-$ DCs excel at MHCII presentation. This feature correlates with higher expression of some cathepsins and other antigen processing enzymes, as well as lower expression of cystatin C (a cathepsin inhibitor) (Dudziak et al., 2007). The superiority of $CD8^-$ DCs over $CD8^+$ DCs for MHCII presentation was evident independent of the mouse strain (C57BL/6, B10.BR or C3H/HeJ), antigen (HEL or OVA) and inflammatory context (with or without co-administration of LPS or anti-CD40) (Fig. 2, 3, 4 and 5). Moreover, $CD8^-$ DCs excel at MHCII presentation whether the antigen is internalized by receptor-mediated endocytosis, pinocytosis or phagocytosis (Fig. 13, 17 and 18).

DCs present antigens efficiently in the steady state. Although DC activation can improve subsequent T cell activation, TLR ligation is not necessary for antigen presentation (Fig. 3 and 4). Interestingly, upon activation, $CD8^+$ DCs improve their ability to activate antigen-specific $CD4^+$ T cells (Fig. 4, 13 and 17). Therefore $CD8^+$ DCs might also have an important and unique role in activating $CD4^+$ T cells in mice undergoing inflammation. The class of effector helper T cells elicited is modulated by the antigen

presenting cell, and $CD8^+$ DCs are known for Th1 polarization (Maldonado-Lopez et al., 1999; Maldonado-Lopez et al., 2001; Soares et al., 2007).

DCs have an intricate relationship with regulatory T cells. There is a feedback regulatory loop between DCs and regulatory T cells: an increase in DC numbers also increases proliferation of regulatory T cells and depletion of DCs diminishes regulatory T cells (Darrasse-Jeze et al., 2009), but depletion of regulatory T cells increases DC proliferation (Kim et al., 2007) through a FL-dependent mechanism (Liu et al., 2009). Distinct DC subsets have unique features that can affect their interaction with regulatory T cells. Consistent with their higher MHCII presentation ability, $CD8^-$ DCs are better at promoting regulatory T cell division. However antigen presentation by $CD8^+$ DCs can convert naïve T cells into regulatory T cells by a TGF-β dependent mechanism (Yamazaki et al., 2008).

With regards to cross-presentation, my data shows that when the amount of antigen captured and the route of internalization are taken into consideration, the two subsets of conventional spleen DCs have similar abilities. When I compared $CD8^+$ and $CD8^-$ DCs for cross-presentation following anti-hDEC targeting, I found that both subsets are equally able to cross-present antigens to $CD8^+$ T cells (Fig. 7 and 13). In agreement with others, there were small differences in MHCI cross-presentation by the two DC subsets in some instances: $CD8^+$ DCs were slightly more efficient than $CD8^-$ DCs, after phagocytosis of OVA-adsorbed beads or soluble antigen (Schnorrer et al., 2006), and also after receptor-mediated endocytosis when limiting number of DCs are compared (Fig. 17,

18 and 14). However, these differences between $CD8^+$ and $CD8^-$ DCs were far less pronounced than the difference between either subset of DCs and other antigen presenting cells. Therefore it seems unlikely that $CD8^+$ DCs possess a specialized cellular machinery that facilitates antigen cross-presentation; given access to antigen, $CD8^-$ DCs are also very efficient at MHCI cross-presentation.

Cross-presentation of cell-associated antigens is important for generating cytotoxic T cells to control viral infections and tumor growth. $CD8^+$ DCs are very efficient to capture dying cells (Iyoda et al., 2002) and this feature enable access to a huge and important source of antigens. Hence, mice deficient in the transcription factor Batf3, which lack $CD8^+$ DCs, have reduced $CD8^+$ T cell responses to West Nile virus and impaired tumor rejection (Hildner et al., 2008).

After antigen targeting to DCs, MHCI presentation lasted for 10 days and MHCII presentation for 5 days (Fig. 2). The difference between MHCI and MHCII is probably due to the distinct sensitivity of the transgenic cells used to detect MHC-pep complexes: OTI cells are more sensitive than OTII cells. Long lasting antigen presentation is most likely sustained by a high initial antigen load and is consistent with DC proliferation and the ability of daughter cells to present MHC-pep complexes from progenitors, as well as the replacement kinetics of splenic DCs (10-14 d) (Liu et al., 2007).

Splenic $CD8^-$ DCs can be further subdivided into $CD4^+$ and $CD4^-$ DCs; however both are phenotypically and functionally similar and regarded as a single group (Edwards et al.,

2003a). In most experiments performed for this thesis, when isolating $CD8^-$ DCs I also sorted for $CD4^+$ cells in order to enrich for $DCIR2^+$ cells and avoid inclusion of potential DC progenitors. Moreover one must ensure that other non-DCs that express CD11c are not isolated in the $CD8^-$ DC pool. For example, activated $Ly6C^+$ monocytes upregulate CD11c; therefore in inflammatory settings inclusion of the Ly6C marker is imperative for separating bona fide $CD8^-$ DCs from activated monocytes. In a recent report it was shown that upon inflammation in the gut, monocytes differentiate into $CD11c^+$ cells that express E-cadherin; thus this molecule might also constitute a useful marker in future studies (Siddiqui et al., 2010). It is important to highlight that after viral infection, without proper gating strategies, NK cells might also be isolated together with $CD8^-$ DCs, resulting in erroneous diminishing conclusions on the antigen presentation potential of $CD8^-$ DCs (Lee et al., 2009).

DEC-205-mediated Internalization

Targeting antigen with anti-DCIR2 to $CD8^-$ DCs leads to poor cross-presentation, but anti-hDEC antigen targeting to the same cells results in efficient cross-presentation (Fig. 7). This result might be explained by quantitative differences in the amount of antigen. Anti-DEC205 delivers more antigen to DCs than anti-DCIR2 (Fig. 7, 8C and results not shown). Supporting this concept, anti-hDEC targeting to $CD8^-$ DC elicits more MHCII presentation than anti-DCIR2 (Fig. 7B). Additionally, DCIR2 internalization is not efficient when compared to DEC-205 (Fig. 8 and 9).

In looking for another receptor that would efficiently internalize antigens, I turned to B cells. B cells possess a specialized receptor for antigen uptake (the BCR), and when activated, B cells can upregulate DEC-205 (Fig. 10). Therefore DEC-205 and BCR-mediated antigen capture were compared for MHC presentation. DEC-205 was as effective as the BCR to promote antigen presentation onto MHCII; and DEC-205-mediated antigen capture was more effective than the BCR to promote antigen cross-presentation into MHCI molecules (Fig. 11).

These results also illustrate that MHCI and MHCII presentation are independent: increasing cross-presentation does not necessarily lead to a decrease in MHCII presentation, and antigen internalization through different receptors can influence antigen processing and presentation. DEC-205 is a receptor that efficiently delivers antigens for both MHCI and MHCII presentation.

Although DEC-205 was more efficient than the BCR to promote cross-presentation, activated B cells were significantly less efficient than DCs at cross-presentation (Fig. 13). Previous reports describing B cell cross-presentation did not compare them to bona fide DCs or accounted for the amount of antigen captured (Robson et al., 2008). Activated B cells were as effective as $CD8^+$ DCs at MHCII presentation when the antigen was delivered by receptor-mediated endocytosis (DEC-205 or BCR), but the same cells were ineffective when the antigen enters the cell by bulk phase endocytosis.

As has been previously documented, antigen uptake was at least 100-fold more efficient when delivered through specific receptors as compared to bulk phase pinocytosis (Jiang et al., 1995). However, when equivalent amounts of ingested antigen are compared, the presentation efficiency of DCs was similar for bulk phase and receptor-mediated endocytosis (Fig. 13 and 17). B cells differed from DCs in that antigens internalized by pinocytosis were presented very inefficiently when compared to receptor-mediated uptake (Fig. 17). Unlike DCs, whose primary function is to initiate immunity, B cells present antigens to recruit specific cognate T cell help. The relative inefficiency at processing and presentation of antigens acquired by bulk phase endocytosis guarantees that T cell help remains focused during an immune response. The B cell receptor is highly efficient at capturing cognate antigens for presentation to T cells (Rock et al., 1984), and thus, only B cells that capture antigen through specific antigen receptors will recruit T cell help. In contrast, the DCs' ability to present a range of antigens acquired by pinocytosis serves to broaden the scope of T cell immunity.

DEC-205 might facilitate cross-presentation due to its intracellular trafficking. DEC-205 and MMR are in the same family of C-type lectins (Jiang et al., 1995), and MMR-mediated antigen capture promotes cross-presentation by retaining antigen in early endosomal compartments (Burgdorf et al., 2007). However, DEC-205 and MMR differ in intracellular localization: contrary to the MMR, most DEC-205 co-localizes with Lamp-1 and MHCII positive compartments, and DEC-mediated antigen internalization promotes MHCII presentation in immature GM-DCs (Mahnke et al., 2000).

Mahnke *et al.* have explored DEC-205 intracellular trafficking, trying to identify amino acid residues in the cytoplasmic tail that guide sorting (Mahnke et al., 2000). $MHCII^{+}$ fibroblasts were transfected with chimeric receptors bearing the external domain CD16 (FcγRIII) fused to the cytosolic tail of MMR or DEC-205. In addition to the wild type sequence, DEC-205 receptors bearing truncations and mutations in the cytoplasmic tail were also analyzed in this heterologous system. The authors show that truncated DEC-205 cytoplasmic tails lacking the residues "FSSVRY" have a defect in internalization that impairs antigen presentation. On the other hand, truncated DEC-205 cytoplasmic tails lacking the residues "EDE", or substitution of those residues for alanines, have normal internalization; yet intracellular DEC-205 delivery into Lamp-1+ compartments and MHCII antigen presentation are both impaired (Mahnke et al., 2000). In this system cross-presentation was not addressed (Mahnke et al., 2000).

Based on the above findings, I was interested in assessing whether particular amino acid residues in DEC-205 cytoplasmic tail sequences might control access to "cross-presentation permissive compartments". In my analysis I used DEC-205 bearing similar cytoplasmic tail truncations and mutations as Mahnke *et al.* (Table 1). However I transduced the full receptor into activated B cells and performed antigen targeting with chimeric monoclonal anti-DEC-OVA for analysis of both MHCI and MHCII presentation.

In accordance with previous results, I also found that a DEC-205 receptor bearing a short cytoplasmic tail missing the "FSSVRY" motif (DEC-short) was defective in

internalization. However it only had a minor impact on antigen presentation (Fig. 12A). This can be attributed to the protocol used for antigen administration: anti-DEC-OVA was added to B cells on ice and excess antigen washed before incubation at 37°C. When I performed similar experiments where anti-DEC-OVA was administered for 2 hours at 37°C, the difference between DEC-WT and DEC–short was much more dramatic (results not shown): DEC-WT can recycle, thus antigen administration at 37°C enables more antigen capture than antigen administration on ice. Even though DEC-short receptors are severely impaired in internalization, over time some antigen is internalized, and it proved to be enough to allow for antigen presentation onto both MHCI and MHCII.

All other DEC-205 receptors bearing cytoplasmic truncations or mutations into alanines had an identical behavior as DEC-WT for MHCII presentation and cross-presentation (Fig. 12). It is hard to reconcile this observation with the conclusions drawn by Mahnke *et al*., which defined the importance of the cytoplasmic tri-acidic cluster "EDE" in promoting MHCII presentation. It is possible that in B cells (and other antigen presenting cells expressing DEC-205), the DEC-205 transmembrane or the extracellular domain associate with other proteins that dictate intracellular sorting. Alternatively, since I used a different DEC-205 ligand (I used a monoclonal antibody and Mahnke *et al.* used polyclonal antibodies), the antigen might dissociate from the DEC-205 receptor in endosomes bearing different characteristics, thus presenting distinct susceptibility to antigen processing and presentation. In conclusion, I could not identify amino acid residues in the cytoplasmic tail of DEC-205 to account for intracellular sorting into

antigen presentation compartments. In my experiments, the main factor affecting antigen presentation was amount of antigen internalized.

The recycling properties of DEC-205 constitute an advantage that increases antigen capture and consequently antigen presentation. This advantage was observed when antigen was administered at 37°C to activated B cells, as discussed above. Similar differences were also observed between DEC-205 and the BCR: when anti-DEC-OVA and NP-OVA where given for 2 hours at 37°C to NP-specific activated B (similar to Fig. 11), DEC-205 promoted higher antigen capture and presentation than the BCR, since the BCR does not recycle (results not shown). These observations are important and should also be taken into consideration when comparing anti-DEC targeting to anti-DCIR2 targeting *in vivo*, since DCIR2 does not recycle (results not shown). In conclusion, recycling provides another advantage of DEC-205 over DCIR2 for antigen delivery

Comparing other APCs to bona fide DCs

Besides DCs, other cell types, such as liver endothelial cells (Limmer et al., 2000) and macrophages (Kovacsovics-Bankowski et al., 1993; Rock et al., 1993), have been reported to cross-present antigens. However a direct comparison between other cell types and DCs, where the amount of antigen internalized is also taken into account, was lacking.

Monocytes develop in the bone marrow and circulate in the blood. The spleen constitutes a reservoir for rapid deployment during inflammation (Swirski et al., 2009). Two subsets can be identified: $Ly6C^{hi}$ monocytes ($CD11b^{+}$ $CCR2^{+}$ $CD62L^{+}$ CX_3CR1^{low}) and $Ly6C^{lo}$ monocytes ($CD11b^{+}$ $CCR2^{-}$ $CD62L^{-}$ CX_3CR1^{high}). The differential expression of chemokine receptors determines their recruitment and might impact function. In the steady state monocytes are precursors of numerous populations of tissue macrophages: liver Kupffer cells, splenic marginal zone and red pulp macrophages, lung alveolar macrophages and microglial cells in the central nervous system. In the steady state, monocytes do not significantly contribute to the pool of DCs in lymphoid organs (Liu et al., 2009) and may only contribute to $CD103^{-}$ populations of $CD11c^{+}$ cells in non-lymphoid organs (Bogunovic et al., 2009; Varol et al., 2009), which have poor T cell stimulation capacity (Bogunovic et al., 2009; Schulz et al., 2009). Monocytes are endowed with great plasticity, and during inflammation, activated monocytes can also differentiate into cells with similar features as DCs. For example, during *Listeria* infection, the spleen is infiltrated by short-lived $CD11c^{+}$ $MHCII^{+}$ monocyte-derived cells that secrete TNF-α and NO (tip-DCs), which are important for innate bacterial clearing (Serbina et al., 2003). Yet activated $CD11c^{+}$ monocytes do not express characteristic DC lectins (DCIR2 or DEC-205) or high levels of Flk2 (FL receptor), but instead express the M-CSF receptor like their monocyte progenitors (Fig. 15 and 19).

Activated monocytes contribute to immunity against several pathogens, such as influenza (Nakano et al., 2009), *Toxoplasma* (Robben et al., 2005) *Lesihmania* (Leon et al., 2007) and *Listeria* (Serbina et al., 2003). However activated monocytes do not appear to be

responsible for the initiation of adaptive immune responses (Serbina et al., 2003). This is consistent with these cells' lower level of MHCII expression (Fig. 15, 16 and 19) and their poor performance in MHCII presentation (Fig. 13, 17 and 18), irrespective of the mechanism of antigen capture, including peptide pulsing.

Activated monocytes can efficiently cross-present antigens captured through DEC-205 or pinocytosis, but not phagocytized antigens (Fig. 13, 17 and 18). Similar results were obtained with GM-DCs, suggesting that even though monocyte-derived cells can cross-present antigens, they might rely on different mechanisms than bona fide DCs. A recent report has shown, for example, that activated monocytes, unlike $CD8^+$ DCs, depend on insulin-regulated aminopeptidase (IRAP) in early endosomes to trim peptides for MHCI loading and cross-presentation (Segura et al., 2009).

A possible role of monocyte-derived cells in T cell responses is a matter of debate. Monocyte-derived cells have been shown to contribute to T cell immunity during *Leishmania* infection (Leon et al., 2007) and *Aspergillus fumigatus* lung infection (Hohl et al., 2009), as well as Th1 polarization in both influenza infection and CFA immunization (Nakano et al., 2009). However, formal proof showing efficient antigen processing and presentation by monocyte-derived cells is still lacking. Inflammatory reactions involve the orchestrated recruitment and activation of different cell types. Therefore the use of CCR2-deficient mice, which have impaired monocyte emigration from the bone marrow, or monocyte depletion systems are not ideal for studying specific

functions carried out by monocytes. Lack of monocyte-derived cells might, for example, impair DC activation or antigen transport to lymph nodes (Ravindran et al., 2007).

Monocytes can undergo distinct activation programs and differentiation, depending on the stimuli, and thereby acquire different functions. Cells similar to tip-DCs also occur naturally in the lamina propria of naïve mice, in response to commensal bacteria, where they induce IgA class switching (Tezuka et al., 2007). Monocytes plasticity has been well explored by *in vitro* systems: whereas addition of M-CSF triggers macrophage-like differentiation, GM-CSF promotes a DC-like differentiation program, generating $CD11c^+$ cells. In the CFA immunization model, activation of monocytes is dependent on GM-CSF signaling (Fig. 15), but in *Listeria* infection, tip-DC differentiation is independent of GM-CSF signaling (results not shown) and triggered by IFN-γ production by NK cells (Kang et al., 2008). Yet both activated monocytes from immunized mice and tip-DCs from *Listeria* infected mice were poor at $CD4^+$ T cell activation and at cross-presentation of phagocytized antigens (Fig. 19). Nevertheless, due to the notable plasticity of hematopoietic cells, it is still conceivable that, given the right cues (e.g. cytokines), monocytes might differentiate into cells that are efficient at T cell activation.

Although they appear to be most closely related to activated monocytes and tip-DCs (Robbins et al., 2008; Xu et al., 2007), the physiologic counterparts of GM-DCs remain to be defined. Similarly to *in vivo* activated monocytes, GM-DCs can present antigens captured by receptor-mediated or bulk phase pinocytosis but are far less efficient in presenting phagocytized antigen than conventional DCs (Fig 13, 17 and 18). This

observation is consistent with the finding that mononuclear phagocytes tend to have more developed lysosomes that may hamper the escape of processed peptides into MHC loading compartments (Ehrenreich and Cohn, 1967; Savina and Amigorena, 2007). Macrophages are highly phagocytic, and it was estimated that OVA bound to 2-3 μm diameter beads is cross-presented 1,000 to 10,000-fold more efficiently than soluble antigen (Kovacsovics-Bankowski et al., 1993). However this comparison was based on the concentration of antigen incubated with macrophages and not the actual amount of captured antigen. Red pulp macrophages are more active than splenic DCs for phagocytosis after intravenous administration of polysterene beads, *Escherichia coli* or *Staphylococcus aureus* (Idoyaga et al., 2009). Likewise, GM-DCs and activated monocytes were more phagocytic than splenic DCs: a higher percentage of cells captured beads and also more beads were taken up per cell (results not shown). Therefore, the primary function of activated monocytes and GM-DCs might be as innate immune effector cells, like neutrophils and macrophages, which destroy most phagocytized antigen (Serbina et al., 2003; Soehnlein and Lindbom, 2010; Xu et al., 2007).

DCs transport MHCII to the cell surface after activation by TLR ligation (Inaba et al., 2000; Pierre et al., 1997; Trombetta et al., 2003; Trombetta and Mellman, 2005). However GM-DCs prior activation did not have a positive impact on their ability to present antigens to T cells (Fig 13). In contrast, FL-DCs resembled conventional spleen DCs in this important respect; antigen presentation was enhanced by activation through TLR ligation: FL-CD8$^+$ DCs performed poorly at antigen presentation without LPS activation (Fig. 13). Also, mirroring their splenic counterparts, FL-CD8$^-$ DCs were better

than FL-CD8^{+} DCs in activating MHCII restricted T cells (Fig 13 and 17). FL-DCs originate from the same progenitor (pre-DCs), rely on the same cytokine (FL) for differentiation and expansion, and are more similar to conventional DCs in antigen processing and presentation.

The bone marrow provides a niche for the maintenance and differentiation of hematopoietic progenitor cells: cell-cell interactions and cell-extracellular-matrix contacts have an important role, in addition to various cytokines (Wilson and Trumpp, 2006). Similarly, when DC progenitors arrive in peripheral lymphoid tissues and non-lymphoid tissues, microenvironment cues guide final differentiation to generate different subsets. Given the complexity of the differentiation process, it is not surprising that *in vitro* cultures of progenitors with cytokine mixtures cannot reproduce the same developmental pathway occurring *in vivo*. Nevertheless, the ability to differentiate DC-like cells *in vitro* from precursors is a very important tool for both basic studies and clinical applications. GM-DCs have a closer resemblance to activated monocytes and tip-DCs; thus FL constitutes a better alternative to obtain dendritic cells from precursors. Fully understanding DC development, and dissecting signaling and transcriptional programs, will impact in the design of new strategies for *in vitro* differentiation of human DCs that will more closely resemble the cells present in different tissues *in vivo*.

Concluding remarks

The division of dendritic cells into subsets is supported by functional specialization: I show here that splenic $CD8^-$ DCs excel at MHCII presentation. However no major differences between $CD8^+$ and $CD8^-$ DCs with regards to cross-presentation were observed when the amount of antigen and route of uptake were taken into account.

Cross-presentation onto MHCI molecules is not restricted to conventional DCs. *In vivo* activated monocytes, for example, were efficient at cross-presenting antigens captured by receptor-mediated endocytosis or pinocytosis. Yet the route of antigen uptake has a major impact on the ability of non-DCs to process antigen for MHC presentation: $CD11c^+$ monocyte-derived cells, including GM-DCs, where far inefficient at processing phagocytized antigen for cross-presentation or MHCII presentation. Therefore DCs differ from all other cells tested in that they excel in antigen presentation irrespective of the route of antigen uptake. This observation is important when analyzing the role of monocyte-derived cells during inflammation. Moreover it has implications for the use of GM-DCs for antigen presentation to T cells. This study was limited to antigen presentation assays, but it would be valuable to extend it to also analyze the development of different classes of effector T cells.

Expression of CD11c and MHCII is not sufficient to classify a cell as a DC and antigen presentation assays should be carefully performed to analyze relative efficiency. The

amount of antigen captured is a major factor that influences the magnitude of antigen presentation and should be more carefully monitored.

Expression of particular patterns in pathogens or antigen targeting to receptors provides antigen access to specific cell types. *In vivo* antigen delivery with anti-DEC antibodies is efficient for antigen presentation because it targets DCs and ensures internalization of high amounts of antigen. $CD8^-$ DEC^- DCs are better at presenting antigen onto MHCII than $CD8^+$ DEC^+ DCs, however upon activation $CD8^+$ DEC^+ DCs increase their MHCII processing ability. Thus, when combined with appropriate maturation stimuli, anti-DEC antigen targeting provides an effective strategy to activate both $CD8^+$ and $CD4^+$ T cells. It would be desirable to identify other receptors that have similar features as DEC but are expressed in $CD8^-$ DCs or both DC subsets.

References

Aida Y. and Pabst M. J. (1990) Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J Immunol Methods* **132**, 191-5.

Amigorena S. and Savina A. (2010) Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr Opin Immunol* **22**, 109-17.

Ardavin C. (2003) Origin, precursors and differentiation of mouse dendritic cells. *Nat Rev Immunol* **3**, 582-90.

Babbitt B. P., Allen P. M., Matsueda G., Haber E. and Unanue E. R. (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* **317**, 359-61.

Bar-On L. and Jung S. (2010) Defining dendritic cells by conditional and constitutive cell ablation. *Immunol Rev* **234**, 76-89.

Bates E. E., Fournier N., Garcia E., Valladeau J., Durand I., Pin J. J., Zurawski S. M., Patel S., Abrams J. S., Lebecque S., Garrone P. and Saeland S. (1999) APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif. *J Immunol* **163**, 1973-83.

Bedoui S., Whitney P. G., Waithman J., Eidsmo L., Wakim L., Caminschi I., Allan R. S., Wojtasiak M., Shortman K., Carbone F. R., Brooks A. G. and Heath W. R. (2009) Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol* **10**, 488-95.

Belizaire R. and Unanue E. R. (2009) Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc Natl Acad Sci U S A* **106**, 17463-8.

Belz G. T., Shortman K., Bevan M. J. and Heath W. R. (2005) CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol* **175**, 196-200.

Bogunovic M., Ginhoux F., Helft J., Shang L., Hashimoto D., Greter M., Liu K., Jakubzick C., Ingersoll M. A., Leboeuf M., Stanley E. R., Nussenzweig M., Lira S. A., Randolph G. J. and Merad M. (2009) Origin of the lamina propria dendritic cell network. *Immunity* **31**, 513-25.

Bonifacino J. T., LM. (2003) Signals for Sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.*

Bonifaz L., Bonnyay D., Mahnke K., Rivera M., Nussenzweig M. C. and Steinman R. M. (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* **196**, 1627-38.

Bonifaz L. C., Bonnyay D. P., Charalambous A., Darguste D. I., Fujii S., Soares H., Brimnes M. K., Moltedo B., Moran T. M. and Steinman R. M. (2004) In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* **199**, 815-24.

Boscardin S. B., Hafalla J. C., Masilamani R. F., Kamphorst A. O., Zebroski H. A., Rai U., Morrot A., Zavala F., Steinman R. M., Nussenzweig R. S. and Nussenzweig M. C. (2006) Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med* **203**, 599-606.

Bozzacco L., Trumpfheller C., Siegal F. P., Mehandru S., Markowitz M., Carrington M., Nussenzweig M. C., Piperno A. G. and Steinman R. M. (2007) DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. *Proc Natl Acad Sci U S A* **104**, 1289-94.

Brasel K., De Smedt T., Smith J. L. and Maliszewski C. R. (2000) Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* **96**, 3029-39.

Brown G. D. (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* **6**, 33-43.

Burgdorf S., Kautz A., Bohnert V., Knolle P. A. and Kurts C. (2007) Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* **316**, 612-6.

Busch D. H., Vijh S. and Pamer E. G. (2001) Animal model for infection with Listeria monocytogenes. *Curr Protoc Immunol* **Chapter 19**, Unit 19 9.

Cambi A. and Figdor C. G. (2003) Dual function of C-type lectin-like receptors in the immune system. *Curr Opin Cell Biol* **15**, 539-46.

Cambi A., Gijzen K., de Vries J. M., Torensma R., Joosten B., Adema G. J., Netea M. G., Kullberg B. J., Romani L. and Figdor C. G. (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *Eur J Immunol* **33**, 532-8.

Caminschi I., Proietto A. I., Ahmet F., Kitsoulis S., Shin Teh J., Lo J. C., Rizzitelli A., Wu L., Vremec D., van Dommelen S. L., Campbell I. K., Maraskovsky E., Braley H., Davey G. M., Mottram P., van de Velde N., Jensen K., Lew A. M., Wright M. D., Heath W. R., Shortman K. and Lahoud M. H. (2008) The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* **112**, 3264-73.

Carter R. W., Thompson C., Reid D. M., Wong S. Y. and Tough D. F. (2006a) Induction of CD8+ T cell responses through targeting of antigen to Dectin-2. *Cell Immunol* **239**, 87-91.

Carter R. W., Thompson C., Reid D. M., Wong S. Y. and Tough D. F. (2006b) Preferential induction of CD4+ T cell responses through in vivo targeting of antigen to dendritic cell-associated C-type lectin-1. *J Immunol* **177**, 2276-84.

Caux C., Massacrier C., Vanbervliet B., Dubois B., Van Kooten C., Durand I. and Banchereau J. (1994) Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* **180**, 1263-72.

Cella M., Engering A., Pinet V., Pieters J. and Lanzavecchia A. (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782-7.

Chen W. J., Goldstein J. L. and Brown M. S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J Biol Chem* **265**, 3116-23.

Collawn J. F., Stangel M., Kuhn L. A., Esekogwu V., Jing S. Q., Trowbridge I. S. and Tainer J. A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* **63**, 1061-72.

Conner S. D. and Schmid S. L. (2003) Regulated portals of entry into the cell. *Nature* **422**, 37-44.

Cook A. D., Braine E. L. and Hamilton J. A. (2004) Stimulus-dependent requirement for granulocyte-macrophage colony-stimulating factor in inflammation. *J Immunol* **173**, 4643-51.

Coombes J. L., Siddiqui K. R., Arancibia-Carcamo C. V., Hall J., Sun C. M., Belkaid Y. and Powrie F. (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**, 1757-64.

Crowley M., Inaba K., Witmer-Pack M. and Steinman R. M. (1989) The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* **118**, 108-25.

Dadaglio G., Nelson C. A., Deck M. B., Petzold S. J. and Unanue E. R. (1997) Characterization and quantitation of peptide-MHC complexes produced from hen egg lysozyme using a monoclonal antibody. *Immunity* **6**, 727-38.

Darrasse-Jeze G., Deroubaix S., Mouquet H., Victora G. D., Eisenreich T., Yao K. H., Masilamani R. F., Dustin M. L., Rudensky A., Liu K. and Nussenzweig M. C. (2009) Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J Exp Med* **206**, 1853-62.

Delamarre L., Couture R., Mellman I. and Trombetta E. S. (2006) Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. *J Exp Med* **203**, 2049-55.

Delamarre L., Pack M., Chang H., Mellman I. and Trombetta E. S. (2005) Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* **307**, 1630-4.

den Haan J. M., Lehar S. M. and Bevan M. J. (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-96.

Dickgreber N., Stoitzner P., Bai Y., Price K. M., Farrand K. J., Manning K., Angel C. E., Dunbar P. R., Ronchese F., Fraser J. D., Backstrom B. T. and Hermans I. F. (2009) Targeting antigen to MHC class II molecules promotes efficient cross-presentation and enhances immunotherapy. *J Immunol* **182**, 1260-9.

Dudziak D., Kamphorst A. O., Heidkamp G. F., Buchholz V. R., Trumpfheller C., Yamazaki S., Cheong C., Liu K., Lee H. W., Park C. G., Steinman R. M. and Nussenzweig M. C. (2007) Differential antigen processing by dendritic cell subsets in vivo. *Science* **315**, 107-11.

Dzionek A., Sohma Y., Nagafune J., Cella M., Colonna M., Facchetti F., Gunther G., Johnston I., Lanzavecchia A., Nagasaka T., Okada T., Vermi W., Winkels G., Yamamoto T., Zysk M., Yamaguchi Y. and Schmitz J. (2001) BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* **194**, 1823-34.

Edelson B. T., Kc W., Juang R., Kohyama M., Benoit L. A., Klekotka P. A., Moon C., Albring J. C., Ise W., Michael D. G., Bhattacharya D., Stappenbeck T. S., Holtzman M. J., Sung S. S., Murphy T. L., Hildner K. and Murphy K. M. (2010) Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8{alpha}+ conventional dendritic cells. *J Exp Med*.

Edwards A. D., Chaussabel D., Tomlinson S., Schulz O., Sher A. and Reis e Sousa C. (2003a) Relationships among murine CD11c(high) dendritic cell subsets as revealed by baseline gene expression patterns. *J Immunol* **171**, 47-60.

Edwards A. D., Diebold S. S., Slack E. M., Tomizawa H., Hemmi H., Kaisho T., Akira S. and Reis e Sousa C. (2003b) Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol* **33**, 827-33.

Ehrenreich B. A. and Cohn Z. A. (1967) The uptake and digestion of iodinated human serum albumin by macrophages in vitro. *J Exp Med* **126**, 941-58.

Engering A., Geijtenbeek T. B., van Vliet S. J., Wijers M., van Liempt E., Demaurex N., Lanzavecchia A., Fransen J., Figdor C. G., Piguet V. and van Kooyk Y. (2002) The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* **168**, 2118-26.

Everts B., Perona-Wright G., Smits H. H., Hokke C. H., van der Ham A. J., Fitzsimmons C. M., Doenhoff M. J., van der Bosch J., Mohrs K., Haas H., Mohrs M., Yazdanbakhsh M. and Schramm G. (2009) Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. *J Exp Med* **206**, 1673-80.

Figdor C. G., van Kooyk Y. and Adema G. J. (2002) C-type lectin receptors on dendritic cells and Langerhans cells. *Nat Rev Immunol* **2**, 77-84.

Fogg D. K., Sibon C., Miled C., Jung S., Aucouturier P., Littman D. R., Cumano A. and Geissmann F. (2006) A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* **311**, 83-7.

Fujikado N., Saijo S., Yonezawa T., Shimamori K., Ishii A., Sugai S., Kotaki H., Sudo K., Nose M. and Iwakura Y. (2008) Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. *Nat Med* **14**, 176-80.

Gallucci S. and Matzinger P. (2001) Danger signals: SOS to the immune system. *Curr Opin Immunol* **13**, 114-9.

Garrett W. S., Chen L. M., Kroschewski R., Ebersold M., Turley S., Trombetta S., Galan J. E. and Mellman I. (2000) Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**, 325-34.

Geijtenbeek T. B., Krooshoop D. J., Bleijs D. A., van Vliet S. J., van Duijnhoven G. C., Grabovsky V., Alon R., Figdor C. G. and van Kooyk Y. (2000) DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nat Immunol* **1**, 353-7.

Geijtenbeek T. B. and van Kooyk Y. (2003) DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission. *Curr Top Microbiol Immunol* **276**, 31-54.

Geissmann F., Gordon S., Hume D. A., Mowat A. M. and Randolph G. J. (2010) Unravelling mononuclear phagocyte heterogeneity. *Nat Rev Immunol* **10**, 453-460.

Geissmann F., Jung S. and Littman D. R. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71-82.

Gilliet M., Cao W. and Liu Y. J. (2008) Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* **8**, 594-606.

Ginhoux F., Liu K., Helft J., Bogunovic M., Greter M., Hashimoto D., Price J., Yin N., Bromberg J., Lira S. A., Stanley E. R., Nussenzweig M. and Merad M. (2009) The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med* **206**, 3115-30.

Greenberg S. and Grinstein S. (2002) Phagocytosis and innate immunity. *Curr Opin Immunol* **14**, 136-45.

Guermonprez P., Valladeau J., Zitvogel L., Thery C. and Amigorena S. (2002) Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* **20**, 621-67.

Guo M., Gong S., Maric S., Misulovin Z., Pack M., Mahnke K., Nussenzweig M. C. and Steinman R. M. (2000) A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum Immunol* **61**, 729-38.

Gurer C., Strowig T., Brilot F., Pack M., Trumpfheller C., Arrey F., Park C. G., Steinman R. M. and Munz C. (2008) Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses. *Blood* **112**, 1231-9.

Hamilton J. A. (2002) GM-CSF in inflammation and autoimmunity. *Trends Immunol* **23**, 403-8.

Hawiger D., Inaba K., Dorsett Y., Guo M., Mahnke K., Rivera M., Ravetch J. V., Steinman R. M. and Nussenzweig M. C. (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* **194**, 769-79.

Hawiger D., Masilamani R. F., Bettelli E., Kuchroo V. K. and Nussenzweig M. C. (2004) Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo. *Immunity* **20**, 695-705.

Heath W. R., Belz G. T., Behrens G. M., Smith C. M., Forehan S. P., Parish I. A., Davey G. M., Wilson N. S., Carbone F. R. and Villadangos J. A. (2004) Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* **199**, 9-26.

Henri S., Poulin L. F., Tamoutounour S., Ardouin L., Guilliams M., de Bovis B., Devilard E., Viret C., Azukizawa H., Kissenpfennig A. and Malissen B. (2010) CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* **207**, 189-206, S1-6.

Henri S., Vremec D., Kamath A., Waithman J., Williams S., Benoist C., Burnham K., Saeland S., Handman E. and Shortman K. (2001) The dendritic cell populations of mouse lymph nodes. *J Immunol* **167**, 741-8.

Hildner K., Edelson B. T., Purtha W. E., Diamond M., Matsushita H., Kohyama M., Calderon B., Schraml B. U., Unanue E. R., Diamond M. S., Schreiber R. D., Murphy T. L. and Murphy K. M. (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* **322**, 1097-100.

Hohl T. M., Rivera A., Lipuma L., Gallegos A., Shi C., Mack M. and Pamer E. G. (2009) Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe* **6**, 470-81.

Hollingdale M. R., Nardin E. H., Tharavanij S., Schwartz A. L. and Nussenzweig R. S. (1984) Inhibition of entry of Plasmodium falciparum and P. vivax sporozoites into cultured cells; an in vitro assay of protective antibodies. *J Immunol* **132**, 909-13.

Idoyaga J., Cheong C., Suda K., Suda N., Kim J. Y., Lee H., Park C. G. and Steinman R. M. (2008) Cutting edge: langerin/CD207 receptor on dendritic cells mediates efficient antigen presentation on MHC I and II products in vivo. *J Immunol* **180**, 3647-50.

Idoyaga J., Suda N., Suda K., Park C. G. and Steinman R. M. (2009) Antibody to Langerin/CD207 localizes large numbers of CD8alpha+ dendritic cells to the marginal zone of mouse spleen. *Proc Natl Acad Sci U S A* **106**, 1524-9.

Inaba K., Inaba M., Romani N., Aya H., Deguchi M., Ikehara S., Muramatsu S. and Steinman R. M. (1992a) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**, 1693-702.

Inaba K., Steinman R. M., Pack M. W., Aya H., Inaba M., Sudo T., Wolpe S. and Schuler G. (1992b) Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* **175**, 1157-67.

Inaba K., Steinman R. M., Van Voorhis W. C. and Muramatsu S. (1983) Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc Natl Acad Sci U S A* **80**, 6041-5.

Inaba K., Swiggard W. J., Inaba M., Meltzer J., Mirza A., Sasagawa T., Nussenzweig M. C. and Steinman R. M. (1995) Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. *Cell Immunol* **163**, 148-56.

Inaba K., Turley S., Iyoda T., Yamaide F., Shimoyama S., Reis e Sousa C., Germain R. N., Mellman I. and Steinman R. M. (2000) The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J Exp Med* **191**, 927-36.

Ito T., Wang Y. H., Duramad O., Hori T., Delespesse G. J., Watanabe N., Qin F. X., Yao Z., Cao W. and Liu Y. J. (2005) TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* **202**, 1213-23.

Iyoda T., Shimoyama S., Liu K., Omatsu Y., Akiyama Y., Maeda Y., Takahara K., Steinman R. M. and Inaba K. (2002) The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med* **195**, 1289-302.

Jensen P. E. (2007) Recent advances in antigen processing and presentation. *Nat Immunol* **8**, 1041-8.

Jiang W., Swiggard W. J., Heufler C., Peng M., Mirza A., Steinman R. M. and Nussenzweig M. C. (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* **375**, 151-5.

Jirmo A. C., Nagel C. H., Bohnen C., Sodeik B. and Behrens G. M. (2009) Contribution of direct and cross-presentation to CTL immunity against herpes simplex virus 1. *J Immunol* **182**, 283-92.

Joffre O., Nolte M. A., Sporri R. and Reis e Sousa C. (2009) Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunol Rev* **227**, 234-47.

Johannes L. and Popoff V. (2008) Tracing the retrograde route in protein trafficking. *Cell* **135**, 1175-87.

Kanazawa N., Tashiro K. and Miyachi Y. (2004) Signaling and immune regulatory role of the dendritic cell immunoreceptor (DCIR) family lectins: DCIR, DCAR, dectin-2 and BDCA-2. *Immunobiology* **209**, 179-90.

Kang S. J., Liang H. E., Reizis B. and Locksley R. M. (2008) Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells. *Immunity* **29**, 819-33.

Kerr M. C. and Teasdale R. D. (2009) Defining macropinocytosis. *Traffic* **10**, 364-71.

Kim J. M., Rasmussen J. P. and Rudensky A. Y. (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* **8**, 191-7.

King I. L., Kroenke M. A. and Segal B. M. (2010) GM-CSF-dependent, CD103+ dermal dendritic cells play a critical role in Th effector cell differentiation after subcutaneous immunization. *J Exp Med*.

Kovacsovics-Bankowski M., Clark K., Benacerraf B. and Rock K. L. (1993) Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U S A* **90**, 4942-6.

Kraal G., Breel M., Janse M. and Bruin G. (1986) Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J Exp Med* **163**, 981-97.

Kretschmer K., Apostolou I., Hawiger D., Khazaie K., Nussenzweig M. C. and von Boehmer H. (2005) Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* **6**, 1219-27.

Kronin V., Vremec D., Winkel K., Classon B. J., Miller R. G., Mak T. W., Shortman K. and Suss G. (1997) Are CD8+ dendritic cells (DC) veto cells? The role of CD8 on DC in DC development and in the regulation of CD4 and CD8 T cell responses. *Int Immunol* **9**, 1061-4.

Lakadamyali M., Rust M. J. and Zhuang X. (2006) Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell* **124**, 997-1009.

Landsverk O. J., Bakke O. and Gregers T. F. (2009) MHC II and the endocytic pathway: regulation by invariant chain. *Scand J Immunol* **70**, 184-93.

Lee H. K., Zamora M., Linehan M. M., Iijima N., Gonzalez D., Haberman A. and Iwasaki A. (2009) Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. *J Exp Med* **206**, 359-70.

Leibundgut-Landmann S., Osorio F., Brown G. D. and Reis e Sousa C. (2008) Stimulation of dendritic cells via the dectin-1/Syk pathway allows priming of cytotoxic T-cell responses. *Blood* **112**, 4971-80.

Lennon-Dumenil A. M., Bakker A. H., Maehr R., Fiebiger E., Overkleeft H. S., Rosemblatt M., Ploegh H. L. and Lagaudriere-Gesbert C. (2002) Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J Exp Med* **196**, 529-40.

Leon B., Lopez-Bravo M. and Ardavin C. (2007) Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* **26**, 519-31.

Limmer A., Ohl J., Kurts C., Ljunggren H. G., Reiss Y., Groettrup M., Momburg F., Arnold B. and Knolle P. A. (2000) Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med* **6**, 1348-54.

Lin M. L., Zhan Y., Proietto A. I., Prato S., Wu L., Heath W. R., Villadangos J. A. and Lew A. M. (2008) Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. *Proc Natl Acad Sci U S A* **105**, 3029-34.

Lindquist R. L., Shakhar G., Dudziak D., Wardemann H., Eisenreich T., Dustin M. L. and Nussenzweig M. C. (2004) Visualizing dendritic cell networks in vivo. *Nat Immunol* **5**, 1243-50.

Liu K., Victora G. D., Schwickert T. A., Guermonprez P., Meredith M. M., Yao K., Chu F. F., Randolph G. J., Rudensky A. Y. and Nussenzweig M. (2009) In vivo analysis of dendritic cell development and homeostasis. *Science* **324**, 392-7.

Liu K., Waskow C., Liu X., Yao K., Hoh J. and Nussenzweig M. (2007) Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* **8**, 578-83.

Liu Y. J. (2005) IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* **23**, 275-306.

Mach N., Gillessen S., Wilson S. B., Sheehan C., Mihm M. and Dranoff G. (2000) Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* **60**, 3239-46.

Mahnke K., Guo M., Lee S., Sepulveda H., Swain S. L., Nussenzweig M. and Steinman R. M. (2000) The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol* **151**, 673-84.

Maldonado-Lopez R., De Smedt T., Pajak B., Heirman C., Thielemans K., Leo O., Urbain J., Maliszewski C. R. and Moser M. (1999) Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo. *J Leukoc Biol* **66**, 242-6.

Maldonado-Lopez R., Maliszewski C., Urbain J. and Moser M. (2001) Cytokines regulate the capacity of CD8alpha(+) and CD8alpha(-) dendritic cells to prime Th1/Th2 cells in vivo. *J Immunol* **167**, 4345-50.

Maraskovsky E., Brasel K., Teepe M., Roux E. R., Lyman S. D., Shortman K. and McKenna H. J. (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* **184**, 1953-62.

Maraskovsky E., Daro E., Roux E., Teepe M., Maliszewski C. R., Hoek J., Caron D., Lebsack M. E. and McKenna H. J. (2000) In vivo generation of human dendritic cell subsets by Flt3 ligand. *Blood* **96**, 878-84.

Massacand J. C., Stettler R. C., Meier R., Humphreys N. E., Grencis R. K., Marsland B. J. and Harris N. L. (2009) Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A* **106**, 13968-73.

Maxfield F. R. and McGraw T. E. (2004) Endocytic recycling. *Nat Rev Mol Cell Biol* **5**, 121-32.

McKenna H. J., Stocking K. L., Miller R. E., Brasel K., De Smedt T., Maraskovsky E., Maliszewski C. R., Lynch D. H., Smith J., Pulendran B., Roux E. R., Teepe M., Lyman S. D. and Peschon J. J. (2000) Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95**, 3489-97.

Mellman I. (1996) Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol* **12**, 575-625.

Mellman I. and Steinman R. M. (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**, 255-8.

Miller J. F., Kurts C., Allison J., Kosaka H., Carbone F. and Heath W. R. (1998) Induction of peripheral CD8+ T-cell tolerance by cross-presentation of self antigens. *Immunol Rev* **165**, 267-77.

Munz C. (2010) Antigen processing via autophagy--not only for MHC class II presentation anymore? *Curr Opin Immunol* **22**, 89-93.

Murphy D. B., Lo D., Rath S., Brinster R. L., Flavell R. A., Slanetz A. and Janeway C. A., Jr. (1989) A novel MHC class II epitope expressed in thymic medulla but not cortex. *Nature* **338**, 765-8.

Nagao K., Ginhoux F., Leitner W. W., Motegi S., Bennett C. L., Clausen B. E., Merad M. and Udey M. C. (2009) Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc Natl Acad Sci U S A* **106**, 3312-7.

Naik S. H., Metcalf D., van Nieuwenhuijze A., Wicks I., Wu L., O'Keeffe M. and Shortman K. (2006) Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol* **7**, 663-71.

Naik S. H., Proietto A. I., Wilson N. S., Dakic A., Schnorrer P., Fuchsberger M., Lahoud M. H., O'Keeffe M., Shao Q. X., Chen W. F., Villadangos J. A., Shortman K. and Wu L. (2005) Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* **174**, 6592-7.

Naik S. H., Sathe P., Park H. Y., Metcalf D., Proietto A. I., Dakic A., Carotta S., O'Keeffe M., Bahlo M., Papenfuss A., Kwak J. Y., Wu L. and Shortman K. (2007) Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* **8**, 1217-26.

Nakano H., Lin K. L., Yanagita M., Charbonneau C., Cook D. N., Kakiuchi T. and Gunn M. D. (2009) Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat Immunol* **10**, 394-402.

Nedjic J., Aichinger M., Emmerich J., Mizushima N. and Klein L. (2008) Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* **455**, 396-400.

Nussenzweig M. C., Steinman R. M., Gutchinov B. and Cohn Z. A. (1980) Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* **152**, 1070-84.

Nussenzweig M. C., Steinman R. M., Witmer M. D. and Gutchinov B. (1982) A monoclonal antibody specific for mouse dendritic cells. *Proc Natl Acad Sci U S A* **79**, 161-5.

Ohl L., Mohaupt M., Czeloth N., Hintzen G., Kiafard Z., Zwirner J., Blankenstein T., Henning G. and Forster R. (2004) CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **21**, 279-88.

Onai N., Obata-Onai A., Schmid M. A., Ohteki T., Jarrossay D. and Manz M. G. (2007) Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol* **8**, 1207-16.

Paccaud J. P., Reith W., Johansson B., Magnusson K. E., Mach B. and Carpentier J. L. (1993) Clathrin-coated pit-mediated receptor internalization. Role of internalization signals and receptor mobility. *J Biol Chem* **268**, 23191-6.

Pack M., Trumpfheller C., Thomas D., Park C. G., Granelli-Piperno A., Munz C. and Steinman R. M. (2008) DEC-205/CD205+ dendritic cells are abundant in the white pulp of the human spleen, including the border region between the red and white pulp. *Immunology* **123**, 438-46.

Paludan C., Schmid D., Landthaler M., Vockerodt M., Kube D., Tuschl T. and Munz C. (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* **307**, 593-6.

Pierre P., Turley S. J., Gatti E., Hull M., Meltzer J., Mirza A., Inaba K., Steinman R. M. and Mellman I. (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* **388**, 787-92.

Platt C. D., Ma J. K., Chalouni C., Ebersold M., Bou-Reslan H., Carano R. A., Mellman I. and Delamarre L. (2010) Mature dendritic cells use endocytic receptors to capture and present antigens. *Proc Natl Acad Sci U S A* **107**, 4287-92.

Pooley J. L., Heath W. R. and Shortman K. (2001) Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J Immunol* **166**, 5327-30.

Porgador A., Yewdell J. W., Deng Y., Bennink J. R. and Germain R. N. (1997) Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* **6**, 715-26.

Probst H. C., McCoy K., Okazaki T., Honjo T. and van den Broek M. (2005) Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* **6**, 280-6.

Pulendran B., Tang H. and Denning T. L. (2008) Division of labor, plasticity, and crosstalk between dendritic cell subsets. *Curr Opin Immunol* **20**, 61-7.

Randolph G. J., Inaba K., Robbiani D. F., Steinman R. M. and Muller W. A. (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* **11**, 753-61.

Ravindran R., Rusch L., Itano A., Jenkins M. K. and McSorley S. J. (2007) CCR6-dependent recruitment of blood phagocytes is necessary for rapid CD4 T cell responses to local bacterial infection. *Proc Natl Acad Sci U S A* **104**, 12075-80.

Robb L., Drinkwater C. C., Metcalf D., Li R., Kontgen F., Nicola N. A. and Begley C. G. (1995) Hematopoietic and lung abnormalities in mice with a null mutation of the common beta subunit of the receptors for granulocyte-macrophage colony-stimulating factor and interleukins 3 and 5. *Proc Natl Acad Sci U S A* **92**, 9565-9.

Robben P. M., LaRegina M., Kuziel W. A. and Sibley L. D. (2005) Recruitment of Gr-1+ monocytes is essential for control of acute toxoplasmosis. *J Exp Med* **201**, 1761-9.

Robbins S. H., Walzer T., Dembele D., Thibault C., Defays A., Bessou G., Xu H., Vivier E., Sellars M., Pierre P., Sharp F. R., Chan S., Kastner P. and Dalod M. (2008) Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* **9**, R17.

Robinson M. J., Sancho D., Slack E. C., LeibundGut-Landmann S. and Reis e Sousa C. (2006) Myeloid C-type lectins in innate immunity. *Nat Immunol* **7**, 1258-65.

Robson N. C., Donachie A. M. and Mowat A. M. (2008) Simultaneous presentation and cross-presentation of immune-stimulating complex-associated cognate antigen by antigen-specific B cells. *Eur J Immunol* **38**, 1238-46.

Rock K. L., Benacerraf B. and Abbas A. K. (1984) Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med* **160**, 1102-13.

Rock K. L., Rothstein L., Gamble S. and Fleischacker C. (1993) Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J Immunol* **150**, 438-46.

Saftig P. and Klumperman J. (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat Rev Mol Cell Biol* **10**, 623-35.

Sallusto F., Cella M., Danieli C. and Lanzavecchia A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182**, 389-400.

Sallusto F. and Lanzavecchia A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179**, 1109-18.

Sancho D., Mourao-Sa D., Joffre O. P., Schulz O., Rogers N. C., Pennington D. J., Carlyle J. R. and Reis e Sousa C. (2008) Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* **118**, 2098-110.

Sano G., Hafalla J. C., Morrot A., Abe R., Lafaille J. J. and Zavala F. (2001) Swift development of protective effector functions in naive CD8(+) T cells against malaria liver stages. *J Exp Med* **194**, 173-80.

Savina A. and Amigorena S. (2007) Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev* **219**, 143-56.

Savina A., Jancic C., Hugues S., Guermonprez P., Vargas P., Moura I. C., Lennon-Dumenil A. M., Seabra M. C., Raposo G. and Amigorena S. (2006) NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* **126**, 205-18.

Savina A., Peres A., Cebrian I., Carmo N., Moita C., Hacohen N., Moita L. F. and Amigorena S. (2009) The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* **30**, 544-55.

Schmid D., Pypaert M. and Munz C. (2007) Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* **26**, 79-92.

Schmid M. A., Kingston D., Boddupalli S. and Manz M. G. (2010) Instructive cytokine signals in dendritic cell lineage commitment. *Immunol Rev* **234**, 32-44.

Schnorrer P., Behrens G. M., Wilson N. S., Pooley J. L., Smith C. M., El-Sukkari D., Davey G., Kupresanin F., Li M., Maraskovsky E., Belz G. T., Carbone F. R., Shortman K., Heath W. R. and Villadangos J. A. (2006) The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci U S A* **103**, 10729-34.

Schulz O., Jaensson E., Persson E. K., Liu X., Worbs T., Agace W. W. and Pabst O. (2009) Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* **206**, 3101-14.

Schulz O. and Reis e Sousa C. (2002) Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology* **107**, 183-9.

Schwickert T. A., Lindquist R. L., Shakhar G., Livshits G., Skokos D., Kosco-Vilbois M. H., Dustin M. L. and Nussenzweig M. C. (2007) In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* **446**, 83-7.

Segura E., Albiston A. L., Wicks I. P., Chai S. Y. and Villadangos J. A. (2009) Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc Natl Acad Sci U S A* **106**, 20377-81.

Serbina N. V., Jia T., Hohl T. M. and Pamer E. G. (2008) Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* **26**, 421-52.

Serbina N. V., Salazar-Mather T. P., Biron C. A., Kuziel W. A. and Pamer E. G. (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* **19**, 59-70.

Shen Z., Reznikoff G., Dranoff G. and Rock K. L. (1997) Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* **158**, 2723-30.

Shih T. A., Roederer M. and Nussenzweig M. C. (2002) Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat Immunol* **3**, 399-406.

Shimonkevitz R., Kappler J., Marrack P. and Grey H. (1983) Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J Exp Med* **158**, 303-16.

Shin J. S., Ebersold M., Pypaert M., Delamarre L., Hartley A. and Mellman I. (2006) Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature* **444**, 115-8.

Siddiqui K. R., Laffont S. and Powrie F. (2010) E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity* **32**, 557-67.

Skokos D. and Nussenzweig M. C. (2007) CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. *J Exp Med* **204**, 1525-31.

Soares H., Waechter H., Glaichenhaus N., Mougneau E., Yagita H., Mizenina O., Dudziak D., Nussenzweig M. C. and Steinman R. M. (2007) A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *J Exp Med* **204**, 1095-106.

Soehnlein O. and Lindbom L. (2010) Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* **10**, 427-39.

Soumelis V., Reche P. A., Kanzler H., Yuan W., Edward G., Homey B., Gilliet M., Ho S., Antonenko S., Lauerma A., Smith K., Gorman D., Zurawski S., Abrams J., Menon S., McClanahan T., de Waal-Malefyt Rd R., Bazan F., Kastelein R. A. and Liu Y. J. (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* **3**, 673-80.

Stanley E., Lieschke G. J., Grail D., Metcalf D., Hodgson G., Gall J. A., Maher D. W., Cebon J., Sinickas V. and Dunn A. R. (1994) Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* **91**, 5592-6.

Steinfelder S., Andersen J. F., Cannons J. L., Feng C. G., Joshi M., Dwyer D., Caspar P., Schwartzberg P. L., Sher A. and Jankovic D. (2009) The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med* **206**, 1681-90.

Steinman R. M. (2007) Dendritic cells: understanding immunogenicity. *Eur J Immunol* **37 Suppl 1**, S53-60.

Steinman R. M. and Cohn Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* **137**, 1142-62.

Steinman R. M. and Dhodapkar M. (2001) Active immunization against cancer with dendritic cells: the near future. *Int J Cancer* **94**, 459-73.

Steinman R. M., Gutchinov B., Witmer M. D. and Nussenzweig M. C. (1983) Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J Exp Med* **157**, 613-27.

Steinman R. M., Hawiger D. and Nussenzweig M. C. (2003) Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711.

Steinman R. M. and Witmer M. D. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* **75**, 5132-6.

Sun C. M., Hall J. A., Blank R. B., Bouladoux N., Oukka M., Mora J. R. and Belkaid Y. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**, 1775-85.

Swiggard W. J., Mirza A., Nussenzweig M. C. and Steinman R. M. (1995) DEC-205, a 205-kDa protein abundant on mouse dendritic cells and thymic epithelium that is detected by the monoclonal antibody NLDC-145: purification, characterization, and N-terminal amino acid sequence. *Cell Immunol* **165**, 302-11.

Swirski F. K., Nahrendorf M., Etzrodt M., Wildgruber M., Cortez-Retamozo V., Panizzi P., Figueiredo J. L., Kohler R. H., Chudnovskiy A., Waterman P., Aikawa E., Mempel T. R., Libby P., Weissleder R. and Pittet M. J. (2009) Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* **325**, 612-6.

Taylor B. C., Zaph C., Troy A. E., Du Y., Guild K. J., Comeau M. R. and Artis D. (2009) TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* **206**, 655-67.

Tezuka H., Abe Y., Iwata M., Takeuchi H., Ishikawa H., Matsushita M., Shiohara T., Akira S. and Ohteki T. (2007) Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* **448**, 929-33.

Tiller T., Meffre E., Yurasov S., Tsuiji M., Nussenzweig M. C. and Wardemann H. (2008) Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* **329**, 112-24.

Trombetta E. S., Ebersold M., Garrett W., Pypaert M. and Mellman I. (2003) Activation of lysosomal function during dendritic cell maturation. *Science* **299**, 1400-3.

Trombetta E. S. and Mellman I. (2005) Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* **23**, 975-1028.

Trumpfheller C., Finke J. S., Lopez C. B., Moran T. M., Moltedo B., Soares H., Huang Y., Schlesinger S. J., Park C. G., Nussenzweig M. C., Granelli-Piperno A. and Steinman R. M. (2006) Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med* **203**, 607-17.

van Die I., van Vliet S. J., Nyame A. K., Cummings R. D., Bank C. M., Appelmelk B., Geijtenbeek T. B. and van Kooyk Y. (2003) The dendritic cell-specific C-type lectin DC-SIGN is a receptor for Schistosoma mansoni egg antigens and recognizes the glycan antigen Lewis x. *Glycobiology* **13**, 471-8.

van Kooyk Y. and Geijtenbeek T. B. (2002) A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol Rev* **186**, 47-56.

Varol C., Landsman L., Fogg D. K., Greenshtein L., Gildor B., Margalit R., Kalchenko V., Geissmann F. and Jung S. (2007) Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* **204**, 171-80.

Varol C., Vallon-Eberhard A., Elinav E., Aychek T., Shapira Y., Luche H., Fehling H. J., Hardt W. D., Shakhar G. and Jung S. (2009) Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* **31**, 502-12.

Waldron J. A., Jr., Horn R. G. and Rosenthal A. S. (1973) Antigen-induced proliferation of guinea pig lymphocytes in vitro: obligatory role of macrophages in the recognition of antigen by immune T-lymphocytes. *J Immunol* **111**, 58-64.

Waskow C., Liu K., Darrasse-Jeze G., Guermonprez P., Ginhoux F., Merad M., Shengelia T., Yao K. and Nussenzweig M. (2008) The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol* **9**, 676-83.

Weigel B. J., Nath N., Taylor P. A., Panoskaltsis-Mortari A., Chen W., Krieg A. M., Brasel K. and Blazar B. R. (2002) Comparative analysis of murine marrow-derived dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses. *Blood* **100**, 4169-76.

West M. A., Wallin R. P., Matthews S. P., Svensson H. G., Zaru R., Ljunggren H. G., Prescott A. R. and Watts C. (2004) Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science* **305**, 1153-7.

Wilson A. and Trumpp A. (2006) Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* **6**, 93-106.

Winzler C., Rovere P., Rescigno M., Granucci F., Penna G., Adorini L., Zimmermann V. S., Davoust J. and Ricciardi-Castagnoli P. (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med* **185**, 317-28.

Witmer-Pack M. D., Olivier W., Valinsky J., Schuler G. and Steinman R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J Exp Med* **166**, 1484-98.

Xu Y., Zhan Y., Lew A. M., Naik S. H. and Kershaw M. H. (2007) Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* **179**, 7577-84.

Yamazaki S., Dudziak D., Heidkamp G. F., Fiorese C., Bonito A. J., Inaba K., Nussenzweig M. C. and Steinman R. M. (2008) CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* **181**, 6923-33.

Yrlid U. and Wick M. J. (2002) Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon Salmonella encounter. *J Immunol* **169**, 108-16.

Ziegler K. and Unanue E. R. (1981) Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *J Immunol* **127**, 1869-75.

Zinkernagel R. M. and Doherty P. C. (1974) Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-2.