Regulatory mechanisms in cross-presentation *in vivo*

Dr. med. (HU) Veronika Lukacs-Kornek

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Institute of Molecular Medicine
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Dr. med. (HU) Veronika Lukacs-Kornek

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1. Summary

Dendritic cells can present exogenous antigen to CD8 T cells using a mechanism termed cross-presentation. This mechanism is important for immune responses against tumors, viruses, intracellular pathogens and autoantigens expressed in peripheral tissues. However, the physiological mechanisms that regulate cross-presentation *in vivo* in particular the endocytosis mechanisms involved in this process are not well understood.

The first aim of this study was to elucidate the role of the mannose receptor (MR), a C-type lectin in cross-presentation. MR deficient mice showed reduced uptake of soluble OVA and diminished activation of CD8 T cells. This was due to the reduced cross-presentation ability of DCs in MR deficient mice, as the MR was only present in the cross-presenting $CD8\alpha^+$ DCs. As opposed to soluble OVA, the cell-associated form of this model antigen did not use the MR to enter the cross-presentation pathways. These data provide first formal evidences that a particular antigen uptake receptor, here the MR can specifically introduce antigen into the cross-presentation pathway. Furthermore, they identify this receptor as a further marker of cross-presenting DCs.

The second aim of the present study was to identify the mechanisms by which soluble antigens are transported from the peripheral organs to draining LNs for subsequent cross-presentation. Proteins smaller than the molecular weight of albumin, such as soluble OVA theoretically may pass the kidney glomerular filter and be concentrated in the tubular compartment. It appeared possible that this physiological process might increase the availability of antigen for the cross-presenting APC. Therefore the kidney - renal lymph node (rLN) system was choosen to investigate antigen transport from organ to draining LN. Several lines of independent evidence indicated that soluble OVA was concentrated in the kidney DCs but was transported in a cell-independent fashion to DC resident in the rLN. Further studies showed that these DCs tolerized specific T cells. This tolerization was independent from steady-state migration of kidney DC to draining LN. These findings identify the rLN as a unique site where antigen is rapidly enriched for T cell activation; provided it is small enough to pass the glomerular filter. This mechanism may contribute avoiding unwanted immune responses against innocuous circulating antigens, for example self-serum or food proteins.

In conclusion the present study revealed new *in vivo* mechanisms in regulating cross-presentation of soluble molecules such as antigen uptake via MR and antigen presentation occurring in the kidney – renal LN system.

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3. Abbreviations

AICD	Activation induced cell death
aOVA	Alexa647-labeled Ovalbumin
APC	Antigen Presenting Cell
BM	Bone marrow
BM-DC	Bone marrow derived dendritic cells
BSA	Bovine Serum Albumin
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CFSE	5,6-Carboxy-Succinimidyl-Fluoresceine-Ester
cLN	Cutaneous lymph node
CLR	C-type lectin receptor
CRD	Carbohydrate recognition domain
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC(s)	Dendritic cell(s)
DCIR2	Dendritic Cell Inhibitory Receptor-2
DC-SIGN	Dendritic-cells specific ICAM-3 grabbing non-integrin
DTR	Diphteria Toxin Receptor
EDTA	Ethylene-diamin-tetraacetate
FITC	Fluorescein-5-isothiocyanate
FRC	Fibroblastic reticular cells
GMB	Glomerular basal membran
HEV	High endothelial venules
HSP	Heat shock protein
ICAM	Intracellular Adhesion Molecule
IL	Interleukin
ITAM	Immunoreceptor tyrosin-based activation motif
ITIM	Immunoreceptor tyrosin-based inhibitory motif
i.v.	Intravenous
JAM	Junctional adhesion molecule

kDC	Kidney dendritic cells
LN(s)	Lymph node(s)
LPS	Lipopolysaccharide
MACS	Magnetic Cell Separation
MFI	Mean fluorescence intensity
MHC I-II	Major Histocompatibility Complex-I-II
MR	Macrophage Mannose Receptor
NK	Natural killer cells
NKT	Natural killer T cells
NX	Nephrectomy
OVA	Ovalbumin
OVA-CFA	Ovalbumin in complete Freund`s adjuvant
PD-1	Programmed death receptor-1
PFA	Paraformaldehyde
PS	Phsophatidyl serin
RAG	Recombination activating gene
rLN	Renal lymph node
s.c.	Subcutaneous
sMR	Soluble Macrophage Mannose Receptor
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
7AAD	7-aminoactinomycin D

4. Introduction

4.1 The immune response

Two major types of lymphoid tissue are relevant for induction of adaptive immunity: primary lymphoid organs like the thymus which is responsible for generation of T cells from precursors and secondary lymphoid organs such as the spleen, lymph nodes (LNs) and Peyer`s patches, which represent sites of immune induction. Naïve T cells simply recirculate between the different secondary lymphoid compartments, continually examining antigen-presenting cells (APC) for the presence of their cognate ligand. Recognition of cognate ligand leads to the activation, proliferation and differentiation of T cells into effector and memory cells. The effector cells after leaving the LNs are capable of entering peripheral tissues and targeting site of inflammation in order to fulfill their immune function (1).

4.2 Dendritic cells

The dominant APC in the body are the dendritic cells (DC). In the mouse, these cells are characterized by the expression of the integrin CD11c. Under steady state conditions, phenotypically immature DCs can be found in peripheral tissues and in secondary lymphoid organs (2). In this state they are highly phagocytic and express low level of MHC II and costimulatory molecules such as CD80, CD86 (3). Upon maturation with different stimuli; such as TLR ligands, inflammatory cytokines or CD40-signals after interaction with T cells; they decrease their antigen uptake capacity and upregulate MHC I, MHC II, CD80, CD86 and can produce proinflammatory mediators like IL-12, TNF-α and IL-6, in order to initiate T cell responses (3, 4). DCs, dependent on their maturation state, surface molecule expression, and their cytokine secretion profile, can dictate the type of immune response they induce. Thus, they can decide whether to promote T helper or regulatory T-cell responses (5, 6). Furthermore, DCs present also antigens against which not immunity but tolerance is induced. These antigens include environmental proteins chronically found for example in the respiratory tracts (7, 8) as well as self-antigens derived from different organs (9-12). Capture

of these antigens under steady state conditions, in the absence of microbial or inflammatory stimuli, allows DCs to control tolerance (13). In addition to their role in adaptive responses, DCs play a critical role in innate immunity. They are active in responding to microbial challenge and can produce numerous different cytokines involved in host defense, such as IL-12 and both type-I and type-II interferons (6). DCs also can activate NK and NKT cells, which are innate lymphocytes that rapidly kill selected targets and produce different cytokines (14, 15).

Dendritic cells are heterogeneous and can be divided into at least six subsets in mice (16-19). Properties of the different subsets are summarized in Fig. 1.

DC subtypes	CD11c	CD8	CD4	CD205	CD11b	B220	Special features
CD8α+ DC	+	+	-	+	-	-	High IL-12 Cross-priming Cross-tolerance
CD8α-CD4+ DC	+	-	+	-	+	-	Most numerous DC in the spleen
CD8α-CD4- DC	+	-	-	-	+	-	High IFNγ
Langerhans cells	+	-/low	-	Very high	+	-	Traffic to lymph node from the skin Present contact sensitizing antigen
Dermal/Interstitial cells	+	-	-	+	+/-	-	In all tissues Traffic to lymph node Prime CD4T cell immunity to tissue infection
Plasmacytoid DC	Low	+/-	+/-	-	-	+	High IFNα Do not look like DC until stimulated

Figure. 1 Mouse DC subsets, surface phenotype and some important properties
(Adapted from Heath WR. *et al*. 2004 Immun. Rev. (19))

The DC subsets are usually separated by their surface receptor expression. DC expressing distinct subset markers are thought to represent distinct cell lineages that are not interconvertable (20). Recently, Shortman *et al.* (17) distinguished three categories differing in functionality and lifetime location: Pre-DCs, inflammatory DCs, and conventional DCs. Dependent on the migratory ability, later differentiation stages were further divided into

lymphoid tissue resident and classical-migratory DC. The impact and regulation of DC migration are discussed below in details.

4.2.1 Dendritic cells are especially abundant in the kidney

The kidney tubulointerstitium contains numerous dendritic cells, whose function is largely unresolved (21, 22). They are located adjacent to the basolateral surface of the tubular epithelium and the peritubular capillaries (21). As was described by Dong *et al*. they could acquire *iv.* injected dextran molecules from the blood and from the ultrafiltrate as well (23). However the functional consequence of this later phenomenon is unclear. Murine kDC were characterized by CD11c, MHC II, F4/80 and CD11b expression, were phenotypically immature and elicited weak allogenic T cell responses (21). *In vitro* study further demonstrated that kidney DCs, isolated from Flt3 treated animal, could promote the generation of $CD25^{+}CD4^{+}IL10^{+}$ regulatory T cells (24). The tolerogenic phenotype of kDCs was additionally investigated in allograft rejection where transfer of freshly isolated kDCs prolonged heart allograft survival (24). Due to the low number of functional studies available, the exact role of these cells is in the kidney under physiological and pathological condition is unknown.

In mouse models, autoantigen, expressed in the kidney, are presented in draining LN and induce T cell tolerance (11, 25), however it is unknown how these antigens reach the kidney draining LN. It has been shown that freshly prepared kDCs expressed CCR1, CCR2, CCR5, CX3CR1 and very low levels of CCR7 (22, 26). LPS stimulation *in vitro* resulted in upregulation of CCR7 (26), suggesting that kDCs may use this chemokine receptor to leave the kidney under inflammatory condition or after maturation. The exact role of chemokine receptors in DC migration *in vivo* needs to be further elucidated.

4.2.1.1 Filtration as a physiological role of the kidney and its filtration barrier

One of the central functions of the kidney is to excrete low molecular weight plasma waste products into the urine while ensuring that useful filtrated molecules are reabsorbed. The filtration of plasma occurs in specialized filtration units called glomeruli. The glomerulus contains a capillary network, which is surrounded by the Bowman's capsule that collects the filtrated primary urine and introduces it into the tubular system (Fig. 2 A, B). The glomerular capillary wall forms a filtration barrier, which consists of three layers: the inner fenestrated vascular endothelium, the glomerular basement membrane (GBM) and the podocyte cell layer. Between the foot processes of podocytes a porous filter is formed, the so called slit-diaphragm (27) (Fig. 2 A, B). This barrier allows free filtration of molecules below of 70 kDa and ensures that cells and molecules above this size are retained in the circulation. In addition to the size the discrimination is based on 3D structure and the charge of the molecules, allowing a greater penetration of neutral and cationic molecules than of anionic ones of the same size (28). The damage of this barrier, as seen in several inherited or acquired diseases, results in proteinuria, which leads to severe glomerulopathies with end-stage renal disease as a final consequence (27).

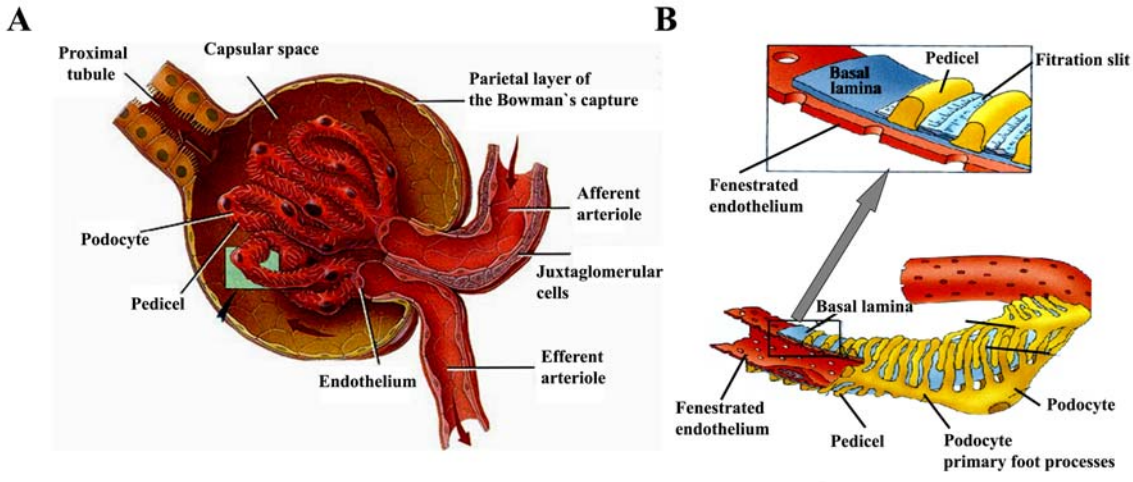

Figure 2. The structure of the filtration barrier of the kidney
A The anatomical structure of the glomeruli.
B The area signed green in A is enlarged. The different layers involved in formation of the filtration barrier are shown: podocytes, basal lamina, and fenestrated endothelium. (Adapted from Berne & Levy Principles of Physiology, (29))

4.2.1.2 The fate of filtrated proteins – the albumin retrieval pathway

Approximately 60 % of the total plasma protein in the serum is albumin. The molecular size of 68 kDa results in filtration of this molecule (30). Therefore albumin has been extensively studied as a model for filterable proteins, in order to follow the fate of such molecules in the kidney. In spite of the daily filtrated load of albumin, which can reach as much as 8 g/day in humans, there are only small amount of this molecule (30mg/day) found in the urine, suggesting more than 99 % of reabsorbtion (30, 31). Reabsorbtion takes place via receptor-mediated endocytosis by the proximal tubular epithelial cells. The reabsorbed albumin undergoes lysosomal degradation into small peptides within minutes in humans and rats which are subsequently released from the basolateral side of the tubular epithelium into the blood stream (28, 31). Understanding the fate and consequence of filtrated molecules in the kidney on the cellular level is especially important since several studies underlined that both under physiological and pathological conditions, albumin (32-34) as well as other filtrated proteins such as growth factors (35) can induce functional responses in kidney tubular cells such as certain chemokine expression which can contribute renal pathology and disease progression. The influences of these events on cellular functions of neighboring non-tubular cells, and the immunological consequences of these processes in healthy and disease animals are largely unresolved.

4.3 Cross-presentation, cross-priming and cross-tolerance

Naive $CD8^+$ T cells recognize antigens in context of major histocompatibility complex-I (MHC I) molecules. Two pathways have been reported to enable DC to process antigens into this pathway for activation of naïve $CD8^+$ T cells. The first is the *classical pathway*: it constitutively facilitates processing of antigens synthesized within the cells for loading on MHC I molecules. The second pathway allows DCs to internalize antigen from the extracellular environment and present them as MHC I bound peptides. In 1976 Bevan *et al*. (36) described this phenomenon for the first time. He immunized mice with allogenic cells and examined the MHC restriction of the induced CTL (cytotoxicity T cell) response. Such immunization generated minor histocompatibility antigen specific CTL that were restricted to both donor and surprisingly host MHC I molecules. The latter was only possible if the host cells acquired donor cellular antigens and processed them on MHC I to induce CTL. This process has been termed as *cross-priming.* The underlying antigen presentation event has later been termed *cross-presentation*. The induction of $CD8^+$ T cell immunity is now generally termed *cross-priming* whereas the induction of $CD8^+$ T cell tolerance is called *cross-tolerance*.

4.3.1 What are the cross-presenting APCs?

4.3.1.1 Dendritic cells

Dendritic cells (DC) have been described to be particularly capable of cross-presentation (37-40). For example, this has been shown by isolating DCs from animals injected with various form of antigen and determining *ex vivo* their ability to induce CD8 T cell responses (37-39, 41, 42). Also it has been shown that DCs exposed to antigen *in vitro* stimulated CD8 T cells upon injection into recipient mice whose resident DCs were incapable of presenting the antigen (43). A further approach is the generation of bone marrow chimeras where only host DCs can present the antigen to $CD8^+$ T cells (44, 45). Microscopy studies showed that during viral infection, proliferating CD8 T cells clustered with cross-presenting DCs within LNs (46).

In spite of this, an important reciprocal experiment; proving the importance of DCs in cross presentation; was made by Jung *et al.* using CD11c-DTR mice where in vivo depletion of DCs inhibited induction of efficient CTL against cell associated antigen (47). However, it has to be kept in mind that some macrophages were also depleted in these mice (48).

4.3.1.2 DC subtypes with distinct ability for cross-presentation?

The first study that addressed which DC subset mediates cross-presentation of cellular antigen came from den Haan *et al*. (37), who identified that $CD8^+$ DC as responsible for cross-priming CTL immunity to OVA-loaded spleen cells. This finding was corroborated by others showing that $CD4^+$ T cells responding to OVA-loaded spleen cells also recognized antigen on the $CD8^+$ cross-presenting DC (40, 49). Furthermore, antigen presentation to $CD4^+$ T and $CD8^+$ T cells by the same DC was required to induce CD4 help ("*cognate help"*) (50). This allows the $CD4^+$ T cell using CD40L to stimulate DCs, which is necessary to induce efficient effector and memory $CD8^+$ T cell responses (51-54). Above data were consistent with the finding that only $CD8\alpha^+$ $CD205^+$ DCs were able to capture apoptotic cell material in the spleen (40, 55). However soluble antigen was shown to be taken up by both $CD8^+$ and $CD8^-$ DC while only the $CD8^+$ ones could cross-present (39, 56). In contrast to this, under certain conditions; such as TLR stimuli (39) or in case of immuncomplexes (38) and peripheral tissue expressed autoantigens (10, 21, 57) cross-priming or cross-tolerance can be induced by not only $CD8^+$ but also by $CD8a^-$ DCs.

4.3.1.3 Macrophages and other cells with cross-presenting abilities

Although DCs are considered the APC most capable of cross-presentation, other cells can also perform this function. For example, macrophages presented antigens on MHC I *in vitro* (58-61) and in adoptive transfer model peptide loaded macrophages are capable of migrating to the draining LN and there directly activating $CD8^+$ T cells (43). However, op/op mice (62) and

mice depleted of macrophages by using toxic liposome (63) did not exhibit substantial defect in initiating adaptive T cell responses.

Other cell types also have been reported to cross-present including B cells (64, 65), human γδ-T cells (66), neutrophils (67, 68) and liver sinusoidal endothelial cells (69). The latter cell type in the liver could cross-present food antigens and induce cross-tolerance (70).

4.3.2 What types of antigens are cross-presented?

Since cross-presentation was discovered there are tremendous evidences for its importance in CD8 T cell responses against tumors, viruses, parasites, intracellular bacteria and autoantigens expressed in peripheral tissues (19, 71, 72). Various forms of antigen can be cross-presented such as soluble proteins, HSP-peptide complexes, immuncomplexes (3, 4). In general cross-priming is biased towards high dose antigens (73) with long half-life (74), and towards cell associated antigens (75), and antigens released during cellular destruction (73). In the case of cross-presentation of cell associated antigen the exact form of antigen transferred from donor cells to the cross-presenting APC is not exactly known. Cross-presentation does not require proteosome activity in the antigen carrying cells (76-78) and as relevant substrate proteolytic intermediates of the proteosome rather than fully processed peptides are considered. This is consistent with studies where expression of the minimal peptides as minigenes in the donor cells did not result in cross-presentation (78, 79). However, under certain *in vitro* conditions peptide transfer through gap junctions from the donor cells to the cross-presenting APC has been described (80).

4.4 Physiological processes regulating availability of antigens for the cross-presenting APC

4.4.1 Antigen uptake

Distinct endocytotic pathways exist to allow internalization of different molecules from the extracellular milieu. APCs like DC use similar pathways found in other cells such as phagocytosis, macropinocytosis, and receptor mediated endocytosis (4).

4.4.1.1 Phagocytosis, macropinocytosis

Particles and soluble antigens are efficiently internalized by phagocytosis and macropinocytosis, respectively. Both processes need actin assembly and formation of pseudopod resulting in the formation of large intracellular vacuoles (81, 82). Phagocytosis denotes the ingestion of large particles or cells > 1μm in diameter and is usually receptor mediated (81). Immature DCs were described to phagocytose bacteria, fungi, and different parasites (3). In the gut epithelium immature DCs projected their dendrites to the apical site of the epithelia and phagocytose bacteria from the intestinal lumen (83). Furthermore DCs phagocytosed apoptotic or necrotic cells, inert particles or liposomes (4) and they could also "bite" pieces of from living neighboring cells in the process termed nibbling (3, 84). In phagocytosis of apoptotic cells αVβ5, αVβ3 integrins (85), complement receptor (CR3) (86), and scavenger receptors like CD36 (87) and PS (phosphatidyl serine) receptor (88) have been implicated. However in DCs no single receptor has been unequivocally proven to be responsible, suggesting they employ parallel or redundant phagocytic receptor systems for the uptake of apoptotic cells, as shown in various knock-out models (89-91).

Macropinocytosis is formed almost exclusively at sites of membrane ruffling, and there is no evidence prooving that receptors are involved (82). This membrane ruffling mechanism is in DCs at least partially regulated by Cdc42-Rac interaction (92, 93) and can be transiently upregulated after TLR stimulation in the early phase of DC maturation (94). In human

monocytes-derived immature DCs, constitutive activity of this process has been proposed, which could reach a rate as high as 40 % of the volume of the cell every hour (95). Macropinocytosis allows cells to internalize large amount of extracellular fluid and fluid dissolved antigen. It has been shown to convey antigens to the MHC II pathway, furthermore under certain in vitro conditions it could lead antigen also to the cross-presentation pathway possibly via allowing access of internalized antigen to the cytosol (96). Nevertheless direct intracellular evidence for such transport via macropinocytosis to the MHC I pathway is still absent. Furthermore, an involvement of this process *in vivo* in the induction of T cell responses remains to be shown.

4.4.1.2 Receptor mediated endocytosis

Receptor mediated endocytosis allows the uptake of macromolecules through specialized regions of the plasma membrane called coated-pits. This process in general initiated by a signal in the cytoplasmic tail of the receptor and leads typically to the formation of clathrin-coated endocytic vesicles (81). To a lesser extent caveolin-coated vesicles can be formed but evidence is scarce that DC use this mechanism extensively (4). Furthermore, DCs can exhibit also a further form of endocytosis that involves neither clathrin nor caveolin (4). Proteins internalized by any of these mechanisms eventually reach the endosomal/ lysosomal compartments, and are loaded on MHC II (4). Many of these receptors; such as Fc- (38, 97), Hsp receptor CD91 (98), complement- (3), scavenger-receptors (99) and members of the C type-lectin family such as DEC205 (13, 100) have been reported to shuttle antigens also into the cross-presentation pathway.

4.4.1.3 C-type lectins

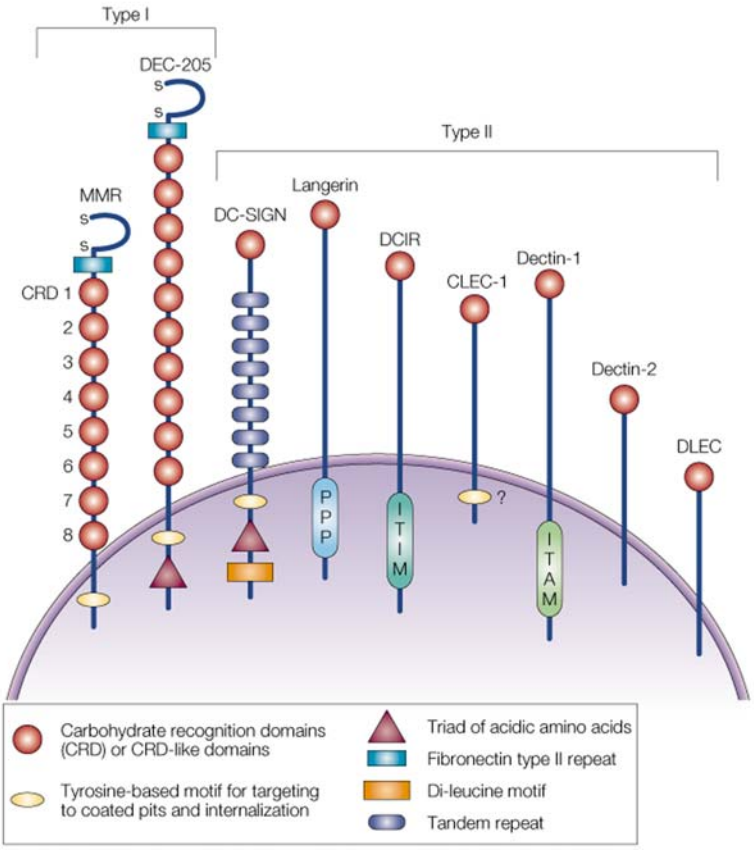

Figure 3. The type-I and type II C-type lectin receptors expressed on dendritic cells
C-type lectins (MMR and DEC-205) contain an amino- terminal cystein-rich repeat (S–S), a fibronectin type II repeat (FN) and 8–10 carbohydrate recognition domains (CRDs), which bind ligand in a Ca_2^+-dependent manner. Type II C-type lectins contain only one CRD at their carboxyl-terminal extracellular domain. The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs involved in antigen uptake: a tyrosine-containing coated-pit intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Other type II C- type lectins contain potential signaling motifs (ITIM, ITAM, proline-rich regions (P)).
CLEC-1, C-type lectin receptor 1; DCIR, dendritic cell immunoreceptor; DC-SIGN, dendritic-cell specific ICAM-3 grabbing non-integrin; DLEC, dendritic cell lectin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MMR, macrophage mannose Adapted from Figdor CC. 2004 (101)

For antigen recognition DCs rely on cell surface receptors to recognize between harmless self-antigens and pathogen-derived antigens. DCs express an array of pattern recognition receptors such as C-type lectin receptors (CLR) and Toll like receptors (TLR) (102). Both are involved

in innate and adaptive immune responses. The term CLR defines carbohydrate-binding molecules that bind ligands in a Ca_2^+-dependent manner. Type I receptors contains extracellularly multiple, type II only one of the carbohydrate recognition domain (CRD) (Fig. 3.).

Using CRD domains, some CLRs recognize N-linked and others O-linked glycosylation sites. Latter structures are often exposed on collagens, mucins and some pathogens whereas N-linked structures are present on the vast majority of glycoproteins in the body as well as on pathogens that use the host's glycosylation for their survival or spread such as retroviruses (102, 103). Although some CLRs recognize monosacharides, like mannose, fucose or galactose, studies showed that the receptor specificity depended on their exact counter structure differing in carbohydrate branching, spacing creating unique sets of carbohydrate recognition profiles. (102, 104, 105) Most CLR receptors function as antigen receptors that are involved in recognition of different pathogens such as bacteria, parasites, fungi and viruses (105, 106). In addition to the role in pathogen recognition under steady state conditions, CLRs are important in the clearance of self-antigen and the induction of tolerance (102). Antigen targeting of CLRs such as DEC-205 *in vitro* and *in vivo* leads to $CD8^+$ T cell tolerance and induction of Tregs (100, 107). The endocytosis mediated by CLRs is guided by their intracellular internalization motifs, whereas some of them contain also ITAM, ITIM (Fig. 3) suggesting potential involvement in signal transduction thereby regulating cellular functions (101). Evidence is emerging that they can also synergize or antagonize TLR signaling like in case of mycobacterial products recognized by DC-SIGN, which induces IL-10, and favors immune escape, antagonizing TLR mediated IL-12 production in DCs (108, 109). In addition to self and non-self antigen recognition, CLRs are important in mediating cell adhesion, homing of leukocytes and even have been shown to provide costimulation during T cell responses (102, 110).

4.4.1.4 The Mannose receptor

The MR family includes MR itself as well as Endo-180, DEC-205 and the phospholipase A_2 receptor. Mannose receptor (MR) is one of the best-characterized type-II CLR. A wilde range of bacteria, yeast and viruses have been described to be recognized by MR (111). (Fig. 4 B) On the molecular level, the receptor recognizes ligands bearing mannose, fucose, N-acetyl glucosamin mediated by its CRDs. (Fig. 4 A) Additionally, via the cystein rich domain, it can bind to sialoadhesin and sulphated carbohydrates (112, 113).

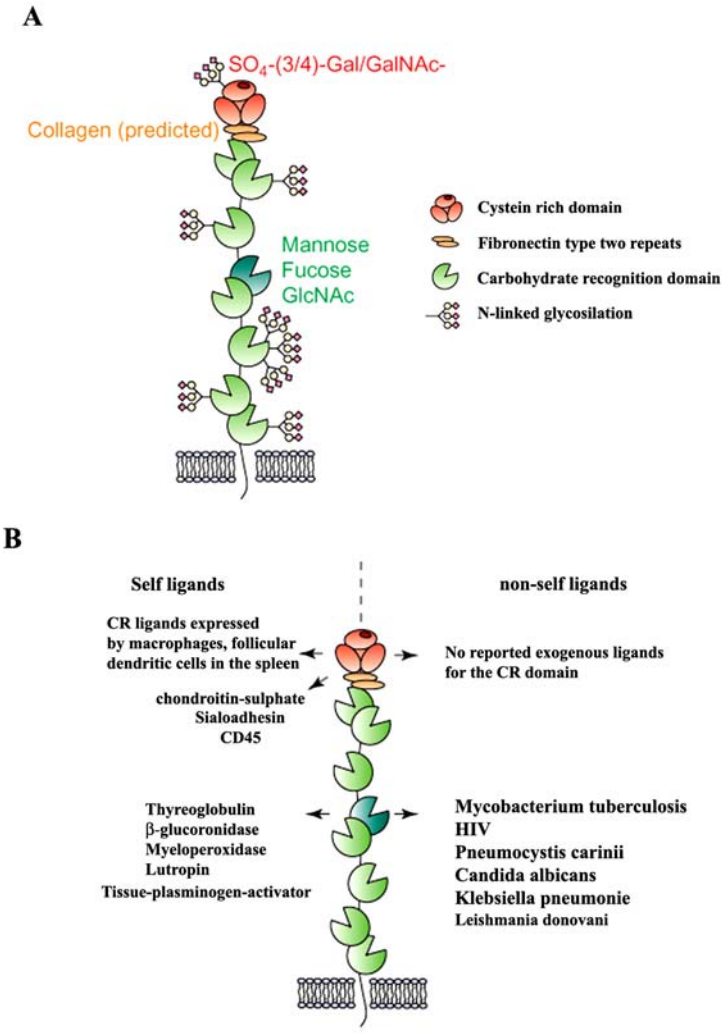

Figure 4. The structure and binding properties of the MR
A The extracellular domain structure of MR included ligands for each of the binding regions
B The MR binds multiple self and non-self ligands through both cystein rich and carbohydrate recognition domain. List of ligands are shown. Modified based on Taylor PR. *et al.* (111)

MR-/- mice, generated by two separate groups (114, 115), allowed determining the requirement of the MR for detecting ligands that had been identified *in vitro*. Surprisingly these mice showed no increased susceptibility to infection against Candida or Pneumocystis carinii (116, 117). Instead, these mice had increased plasma levels of endogenous proteins such as lysosomal hydrolyses (114) and a defect in clearance of luteotropin, latter responsible for reduced litter size in these animals. Additionally, the MR was involved in the clearance of potentially harmful endogenous inflammatory glycoproteins like lysosomal acid phosphatase (118) or the neutrophil derived myeloperoxidase (119). These phenotypes revealed a functional redundancy between CLRs *in vivo* and highlighted the difficulties to predict MR contribution in host defense from *in vitro* ligand binding studies.

In spite of the numerous ligands identified for MR it functions not only as a simple scavenger receptor but also could mediate cell adhesion of lymphocytes through L-selectin (120).

So far most studies have addressed the function of the MR in macrophages, where it is expressed most abundantly (101). In addition to this, MR has also been detected in liver endothelial cells, dermal microvascular endothelial cells, monocytes, Langerhans cells and dendritic cells (101).

4.4.1.5 MR and antigen presentation in DC

The MR has been described as a broad specificity antigen receptor in human monocytes-derived DCs to concentrate macromolecules in MHC II compartment (95, 121). Further involvement in antigen presentation was reported *in vitro* by targeting the MR on human DC with mannosylated BSA or anti-MR mAb fused with tumor antigen, which led to internalization and presentation to T cells in the context of both MHC I and II (121, 122). Additionally mannosylated protein antigen resulted a 200-10.000-fold increase in antigen-presentation of human DCs on MHC II compared to non-mannosylated ones (123). These *in vitro* findings encouraged some groups to designe vaccine using mannosylated tumor-antigens in order to induce anti-tumor responses (124, 125). Some of these vaccines are currently in clinical trial phase I (126). However, when interpreting these studies it has to be considered that mannan or mannosylated proteins which bind with high affinity to MR, also bind to other

C-type lectins such as DC-SIGN (127), that might also have contributed to antigen presentation.

The subcellular localization of MR-internalized cargo revealed that it exclusively localized in early endosomes and was excluded from MHC II^+ compartments (121, 123, 128). Others demonstrated colocalisation of the MR with $MHCII^+$ $CD1b^+$ vesicles, suggesting involvement also in glycolipid presentation (129). The majority of cellular MR was found within the endocytotic pathway and only 15-30 % of the cellular pool located on the cell surface (130). Thus, it was suggested that the MR functions as a fast uptake receptor facilitating rapid internalization of different glycosilated antigen, which can become available for processing and presentation. An unresolved question was the involvement of MR in cross-presentation and its effect on T cell activation *in vivo*. Direct evidence for these functions is limited and contraversial. First of all there is a disagreement on the expression of MR *in vivo*. In the murine system, it was not expressed in T cell areas of secondary lymphoid organs such as the spleen and LNs, which argued against an involvement of the MR in the induction of T cell responses (131). Besides the cell-associated form of the MR, some studies detected a soluble from of this receptor (sMR) in the supernatant of human DC cultures and in the murine serum (132). The functional relevance of this finding is unclear. *In vivo* studies using a construct containing the cystein rich domain of the MR fused with the Fc portion of human IgG identified MR ligands *in vivo* in marginal metallophillic macrophages of the murine spleen, in follicular DC and in subcapsular macrophages of the LNs (133). Although such interactions between MR^+ cells or sMR targeted to CR-ligand-bearing cells suggested an influence on immune responses, formal evidence supporting this notion is scarce.

Upon stimulation with OVA in Alum, CR^+ cells in the spleen showed DC morphology, expressed CXCR5 surface marker and were able to enter the B cell follicles. In addition, after sorting, these cells could present antigen to T and B cells in vitro (134, 135). These findings suggested that MR^+ cells might transport antigen to the places where adaptive immunity is induced. Alternatively, antigen bound to sMR could be targeted to CR^+ cells, implying that the MR could connect innate and adaptive immunity.

The cytoplasmic tail of the MR shows a lack of signaling motifs such as ITAM or ITIM, therefore it is not expected that antigen uptake would lead to DC activation or cytokine release. However, it has been reported that anti-MR antibodies or mycobacterial glycoproteins

matured human DC, resulted in IL10 production, and induced a regulatory phenotype in co-cultured T cells (136, 137). Such anti-inflammatory effects were seen also with LPS, implying a possible cross talk between MR and TLR4 (137). Other ligands such as mannoprotein derived from Cryptococcus neoformans induced efficient IL-12 production and DC maturation in human DCs (138). The above findings raise the question whether the MR could be associated with different adapter molecules in order to alter cellular functions, but this possibility is speculative at present.

4.4.2 The lymphatics and the conduit system

The lymphatic capillaries are blind-ending vessels located in various organs, consisting of endothelial cells and basement membrane. These capillaries converge to lymphatic vessels, surrounded by smooth muscle cells, facilitating an intrinsic pump activity of the vessel. This pump together with extrinsic factors such as a movement of surrounding tissue itself maintains the lymphatic flow. Additionally, valves can be found in bigger lymphatic vessels, which guarantee unidirectional flow. The transported lymph enters the draining LN from the afferent lymph vessel into the subcapsular sinus. (Fig. 5)

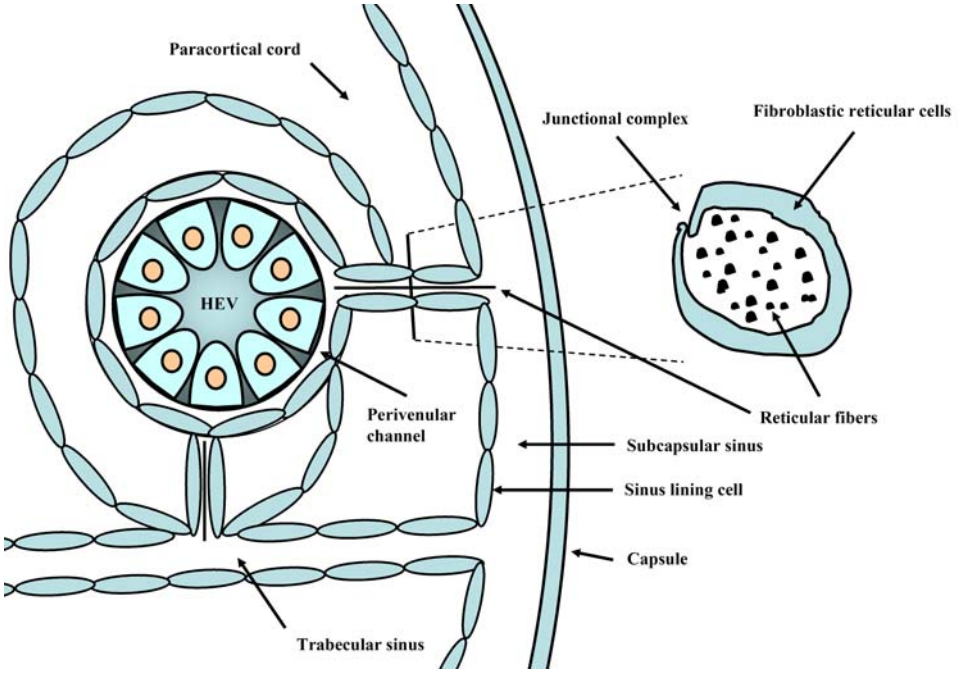

Figure 5. The structure of the lymphatic conduit system
Schematic diagram on the left shows the structural component of the lymph node. FRC conduit starts from the subcapsular sinus and drains lymph to the perivenular channel of the HEV. On the right side of the diagram it is shown how the FRCs envelop the reticular fibers forming channels for the lymphatic flow. Adaptation based on von Adrian *et al.* (139)

From here most of the lymph is channeled through trabecular sinuses across the parenchyma towards the medulla and from there to an efferent lymphatic vessel, which finally leaves the LN (139, 140). Some of the lymph enters the intranodal lymphatic channels. These consist of fibroblastic reticular cells (FRCs) and network of reticular fibers. FRCs envelop these reticular

fibers, forming channels that project from the subcapsular sinus into the T cell area (Fig. 5). This, the so-called conduit system (139, 141), functions primarily as a size exclusion channel allowing small molecules such as chemokines, or injected tracers to reach the T cell zone, the perivenular channels and finally the HEV. Conduit systems as described above were found not only in LNs (141) but also in the thymus (142) and in the spleen (143). The function of this system is to transport chemokines, both homeostatic (CCL19, 21) (144, 145) and inflammatory such as CCL2 (146), arriving with the lymph from the periphery and further target them to the HEV. Thus, it is functioning as a remote control of the peripheral tissue for leukocyte recruitment to LN. This system however also allows efficient transport of soluble tracers or injected antigens arriving with the lymph flow into the T cell/DC zones (147-150). These molecules can accumulate in resident dendritic cells of the T cell area (150). Itano *et al.* have shown a physiological consequence of this system that antigen arriving into LN DC independently via the conduit induced functionally different $CD4^+$ T cell responses compared to antigens carried by DC (149). In spite of studies using artificial settings, such as *s.c.* injection of high amount of tracers or antigens, (148-150) evidence is scarce that under physiological conditions such antigen transport occurs and how it might contribute to T cell responses.

4.4.3 Migration of dendritic cells

Current authoritative reviews distinguish two DC subtypes with distinct migratory properties inside the secondary lymphoid organs (17). One is lymphoid tissue resident non-migrating DCs, which exhibit immature phenotype. It can be further divided into $CD8^+$ and $CD8^-$ DC subpopulations. They take up antigen arriving in the LN via the lymph or via migrating DC. In viral infection (151), migratory DC can hand over antigen to draining LN-resident DC for presentation to T cells, in order to induce immunity (57, 151). These organ derived migratory DC represent the other of the two groups of DCs: They sample antigen in peripheral tissues and transport it to draining LN in order to induce T cell response, either directly or indirectly by transferring antigen to LN-resident DC (17). Migration is triggered by microbial stimuli or by inflammatory cytokines (152). Evidence for such triggered migration has been presented in

the skin, the gut, and the lung (8, 153, 154). After exposure to inflammatory stimuli tissue DCs undergo maturation, and upregulate chemokine receptors such as CCR7, CXCR4, and CCR4 (155). The major chemokine receptor implicated in migration of DC to draining LNs is CCR7 (156). In CCR7 deficient mice DC migration from the skin, gut, and lung was reported to be severely impaired (8, 153, 154, 156). In order to reach the draining LN, DCs first have to enter the lymphatics possibly via adhesion molecules such as ICAM-1, JAM-1 (157). DCs migrate towards CCL21-leu and CCL19 expressed inside of the lymphatic vessels, resulting in their arrival in the subcapsular sinus of the draining LN (140, 157). There they are further directed towards the paracortex by CCL21-ser, CCL19 derived from stroma cells and the HEV in order to reach the place of the induction of T cell activation (140). Maturing DCs can also produce CCL19, which can induce signaling involved in rearrangement of the cytoskeleton (155, 158). Therefore it is possible that DCs use also autocrine mechanisms for CCR7-dependent migration. However the expression of CCR7 alone is not sufficient for DC migration. Other signals such as lipid mediators, cysteinyl leukotriens, prostaglandin E_2, and CD38 are required to sensitize CCR7 to its ligands CCL19 and CCL21 (157). Possibly these mediators alter intracellular signaling pathways, but the exact mechanisms are unclear.

Additional factors independent from the CCR7 were also described to influence DC migration including SP1 (159), histamine, adenosine, and prostaglandin-D_2 (157). The latter two are important in counterbalancing migratory properties of DCs (157).

In the absence of inflammation, constitutive migration of immature DC to organ-draining LN has been observed, for example in the skin (153). Such "steady-state" DC migration is thought to mediate T cell tolerance against organ-derived antigens (13). Tolerogenic cross-presentation of antigen to $CD8^+$T cells (cross-tolerance) has been shown to apply to pancreatic or kidney self antigen in the respective organ-draining LN (11). It is unresolved whether cross-tolerizing DCs were migratory or whether LN-resident $CD8^+$ DC after capturing antigen from organ derived steady-state migrating ones mediated tolerance. In such steady state migration CCR7 was also implicated (153).

4.5 The outcome of cross-presentation – tolerance vs. immunity

Heamatopoetic T cell precursors migrate to the thymus where they undergo positive and negative selection based on their TCR interactions with MHC-peptide complexes, resulting in deletion of self-reactive T cells (*Central tolerance*). Despite of thymic tolerance, healthy individuals harbor self-reactive T cells in the periphery, but these are controlled by so-called *peripheral tolerance* mechanisms acting either directly on the self-reactive T cells (*T cell intrinsic*) or indirectly via additional cells (*T cell extrinsic*) such as T regulatory cells (160). One of the peripheral tolerance mechanisms is the so-called T cell ignorance, which denotes a situation where self-antigen is not accessible (160) or because the amount of antigen does not reach the required threshold (73) to trigger T cell response. Encountering self-antigen by T cells might alternatively lead to functional inactivation, termed anergy. Anergic T cells are characterized by the lack of cytokine production like IL-2, IFN-γ upon restimulation. It has been proposed that the lack of costimulation such as CD80, CD86 is responsible for the induction of T cell anergy (160). Later it became obvious that many other signals delivered through various receptors involving for example CTLA-4, PD-1 (161) contribute to this process (162, 163). Beside this, many signaling and adaptor molecules have been proposed to cause T cell unresponsiveness (164-167).

When T cells become fully activated there are still opportunities to induce tolerance. One possibility is the *phenotypic skewing* when the nature of the response is directed to avoid the pathogenic effects like in case of polarization of T cell response towards T_H-2 cells. Such T_H-2 responses have been linked with down regulation of autoimmunity in diabetes or EAE (Experimental Autoimmune Encephalitis) (160). Also regulation of chemokine receptor expression might serve to prevent autoimmunity in the periphery (168).

Autoimmunity induced by fully activated T cells can be prevented by deletion of the given T cell clones via activation induced cell death (AICD). This mechanism was reported in many models where autoantigen was expressed in peripheral tissues such as in the pancreas, kidney or gut (11, 169, 170). Such peripheral deletion in case of $CD8^+$ T cells (cross-tolerance) involves CD95-signaling (171) but is mainly mediated by Bim-dependent, Bcl-2 inhibitable apoptosis (172).

What determines tolerance vs. immunity and what form of tolerance is used is under intense investigation. It has been suggested that the strength of TCR signaling determines that anergy vs. deletion of CD8 T cells occur. Typically weak, but persistent TCR stimulation causes deletion (170, 173). The importance of chronic persistence of antigens has been demonstrated by showing that tolerized T cells could survive *in vivo* once they were removed from the antigen bearing host (174). Other survival factors, cytokines such as IL-7, IL-2 were proposed to be important for CD8 T cell survival resulting efficient effector and memory responses (175-177). The activation status of the antigen presenting cells is generally thought to represent a major component in self/non-self discrimination. There are numerous reports where the same antigen induced different responses dependent on the presence of adjuvant or TLR ligands (100, 107, 178). Accordingly, under steady state conditions continuous presentation by immature /resting DC induced peripheral tolerance (13, 173).

In addition to DC, liver sinusoidal endothelial cells also can induce efficient CD8 T cell tolerance (70). Recently an additional mechanism was reported, which depended on Aire-gene expressing LN-resident stomal cells that expressed self-antigens themselves and induced $CD8^+$ T tolerance (179).

4.5.1 Tolerance towards soluble antigens

Cross-tolerance may be required also against certain non-self proteins, such as innocuous self-serum or food proteins or orally administered antigen. Systemic injection of soluble antigen devoid of inflammatory stimuli has been widely used to study such T cell tolerance. Most studies have focused on the effect of systemically injected peptides rather than full-length protein antigens on T cell tolerance. Such peptides resulted in thymic and peripheral T cell-apoptosis (180). Further characterization of this phenomenon demonstrated that repeated injection of high dose peptide induced anergy whereas repeated injection of low doses resulted in clonal deletion. Single injection of low dose peptide antigen could induce only incomplete deletion of CD8 T cells (170, 174). Systemic injection of full-length protein antigen have been demonstrated to result in mmunological unresponsiveness (178, 181). Cross-tolerance against blood-borne soluble antigen is thought to be induced in the spleen, because cross-presentation

of such antigen is particularly efficient in this organ (75), but not in LNs (182). Formal proof of tolerance against soluble antigen has been provided for the liver (69).

5. Aim of this study

Part 1

Investigating the role of C-type lectins in antigen uptake

Several receptors mediating antigen uptake in DC have been identified, such as the Fc-receptors, DC-SIGN and DEC-205. A role of the MR in antigen uptake and presentation by DC has been proposed based on the finding that mannosylated proteins are presented more efficiently than non- mannosylated ones (123). It is unclear; however, whether this uptake was due to the MR, as DCs express other receptors, such as DC-SIGN, with affinity for mannosylated proteins. The first aim of this study was to overcome these limitations with the use of MR-/- mice, in order to elucidate the role of the MR in the uptake and presentation of soluble vs. cell-associated ovalbumin (OVA) *in vivo*.

Part 2

Handling of soluble filtrated molecules by kidney dendritic cells

An unresolved question in the field of antigen presentation is how tissue-derived innocuous self-antigens are transported to draining LNs for DC-mediated induction of T cell tolerance. Preliminary work in the lab of Prof. Kurts has shown that the kidney enriches innocuous circulating protein antigens below albumin molecular size (68 kDa). Such molecules can pass the glomerular filter and reach the tubular lumen. To prevent their loss with the urine, they are reabsorbed by tubular epithelial cells and released in degraded form into peritubular capillaries. Recently, it was shown that filterable dextran molecules are not only taken up by tubular epithelial cells, but also by kidney DCs (23). However how the kidney and its dendritic cells handle these filtrating soluble molecules immunologically, whether filtrated low molecular weight antigens reach the draining LN and what consequences they have for T cell activation are unknown. These questions were addressed in this study.

6. Materials and Methods

6.1 Materials

6.1.1 Technical equipment

AutoMACS	Miltenyi, Bergisch Gladbach
Cell culture dishes	25cm^2, 80cm^2, (Nunc, Wiesbaden)
Centrifuge tubes	15ml, 50ml (Greiner Labortechnik, Frickenhauser)
Cover slip (18 x 18 mm)	Menzel Gläser, Braunschweig
Dako Pen	Dako Cytomation GmbH, Hamburg
Flow cytometer	FACS-Canto und LSR II (BD, Heidelberg)
Injection needle	25G, 27G (BD, Heidelberg)
MACS -separator	Miltenyi, Bergisch Gladbach
MACS - Column	MS$^+$ und VS$^+$ (Miltenyi, Bergisch Gladbach)
Metal zip	University of Bonn
Multipipette	Dunn Labortechnik
Microwave	Bosch, Stuttgart
Microscope	IX71 (Olympus, Hamburg)
Neubauer chamber	Brand, Wertheim
Parafilm	American National Can TM, Greenwich
Pasteur pipettes	150mm, 230mm (Roth, Karslruhe)
PD-10 column	Amersham, Uppsala
Petri-dish	10 cm Ø (Nunc, Wiesbaden)
Pipettes	Gilson, Heidelberg
Pipette-boy	Hirschmann Labortechnik, Eberstadt
Reaction tubes	0.5-2mL (Eppendorf, Hamburg)
Scalpel	Aesculap, Tuttlingen
Slide	‘Super Frost Plus’ (Menzel-Gläser, Germany)
Suture material-absorbable	Maxon HR13 (1m, USP 5/10) (Aesculap, Tuttlingen)
Sterile filter	0.2µm (Nunc, Wiesbaden)

UV-irradiation	CL-1000 UV Cross-linker (Upland, USA)
Zip, Nylon-	70µm und 100µm (BD, Heidelberg)

6.1.2 Chemicals and reagents

Acetic acid ($C_2H_4O_2$, MW=60,05)	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Albumin, Bovine serum- (BSA)	Gerbu, Gaiberg
Alexa647 labeling kit	Molecular probes, Netherlands
Ammoniumchlorid (NH_4Cl, MW = 53,49)	Merck, Darmstadt
Brefeldin A ($C_{16}H_{24}O_4$, MW = 280,4)	Sigma, Taufkirchen
Bromphenolblue ($C_{19}H_{10}Br_4O_5S$, MW = 670,0)	Sigma, Taufkirchen
Collagenase A	Roche, Mannheim
CFA (Complete Freund`s Adjuvant)	Sigma, Taufkirchen
CFSE	Molecular Probes, Netherlands
Dimethylsulfoxid (DMSO) ($(CH3)_2OS$, MW = 78,13)	Merck, Darmstadt
Dinatriumhydrogenphosphat (Na_2HPO_4, MW = 142,0)	Merck, Darmstadt
DNAse I	Roche, Mannheim
DQ-OVA	Molecular probes, Netherlands
Ethanol, absolute (C_2H_5OH, MW = 46,07)	Merck, Darmstadt
Ethidiumbromid ($C_{21}H_{20}N_3Br$, MW = 394,3)	Sigma, Deisenhofen
Ethylendiamintetraacetate (EDTA) ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$, MW = 372,2)	Gerbu, Gaiberg
Foetal calf serum (FCS)	PAA, Cölbe
Foetal calf serum (low LPS, low IgG) (FCS^{-LPS})	PAA, Cölbe
L-Glutamine (200mM) ($C_5H_{10}N_2O_3$, MW = 146,1)	Invitrogen, Karlsruhe
HCl, MW = 36,46)	Sigma, Taufkirchen
Heparin	Amersham Pharmacia, Freiburg
HEPES 1 M ($C_8H_{18}N_2O_4S$, MW = 238,3)	Invitrogen, Karlsruhe
Hoechst 33342	Molecular probes, Netherlands

Isopropanol ($(CH_3)_2CHOH$, MW = 60,1)	Merck, Damrstadt
Ketamin	Pharmacia GmbH, Karlsruhe
Lymphoprep	PAA, Cölbe
2-Mercaptoethanol ($HS(CH_2)_2OH$, MW = 78,13)	Sigma, Taufkirchen
Natriumazid (NaN_3, MW = 65,01)	Sigma, Taufkirchen
Natriumbicarbonat ($NaHCO_3$, MW = 84,01)	Sigma, Taufkirchen
Natriumchlorid (NaCl, MW = 58,44)	Merck, Darmstadt
Natriumchlorid (NaCl) 0.9%	B.Braun, Melsungen
Natriumdihydrogenphosphat (NaH_2PO_4, MW = 120,0)	Merck, Darmstadt
Natriumhydrogenphosphat (Na_2HPO_4, MW = 142)	Sigma, Taufkirchen
Natriumhydroxid (NaOH, MW = 40,0)	Merck, Darmstadt
Ovalbumin, Grad V	Sigma, Taufkirchen
Paraformaldehyde (PFA) ($H(-OCH_2)_n-OH$)	Serva, Heidelberg
PBS	Biochrom, Berlin
Percoll	Amersham Pharmacia, Freiburg
Propidium iodide	Molecular probes, Netherlands
Protein assay kit	Pierce, Bonn
Rompun	aniMedica GmbH, Senden-Boesensell
RPMI	Invitrogen, Karlsruhe
Saponin	Sigma, Taufkirchen
SIINFEKL	Abcam, Berlin
Triton X-100	Sigma, Taufkirchen
Trypanblue, 0,4% ($C_{34}H_{24}N_6O_{14}S_4Na_4$, MW = 960,8)	Biochrom, Berlin
Trypsin/EDTA	Invitrogen, Karlsruhe
7AAD (7-Aminoactinomycin D)	Sigma, Taufkirchen

6.1.3 Buffers and media

PBS

PBS: NaH_2PO_4 20 mM, NaCl 50mM, Ph adjusted to 7.4. It was autoclaved and stored at 4 °C.

PBS containing 0.1 % bovine serum albumin (BSA)

It was prepared by dissolving 0.1 g BSA (> 98 % purity, Serva, Heidelberg) in 100 ml PBS. The solution was sterilized by filtration through a 0.45µm sterile syringe filter.

FACS buffer

It consisted of PBS containing 0.1 % BSA and 0.01 % NaN_3. Solution was stored at 4°C.

MACS buffer

It consisted of PBS containing 0.1 % BSA, 2mM EDTA. Solution was stored at 4°C.

Red blood cell removal buffer (RCRB)

It was prepared by dissolving 15.58 g NH_4Cl (0.146 M), 0.074 g ethylenediamine-tetra-acetic acid (EDTA)-disodium salt, 2.0 g $NaHCO_3$ in 2 litres of Milli Q water. Final pH = 7.3. RCRB was usually pre-warmed to 37°C before use.

4 % acetic acid solution

It was prepared by mixing 40 ml glacial acetic acid with 960 ml double distilled water. This solution was not sterilized.

Carb/bicarb buffer (0.5 M)

It was prepared by adding 4.4 ml of Na_2CO_3 solution (5.3 g in 100 ml pure water) to 100 ml $NaHCO_3$ solution (4.2 g in 100 ml pure water). PH adjusted to 9.0.

20 % Triton X-100

Solution was prepared by mixing 20 ml Triton X-100 with 80 ml Milli Q water. This solution was not sterilised.

EDTA 0.5M

186.1g Ethylendiamintetraacetat (EDTA) ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$, MW = 372,2) was solved in 800 ml Milli Q water. Ph was adjusted to 8.0 with NaOH than filled up with Milli Q water till 1000 ml. Solution was autoclaved and stored at room temperature.

20 % PFA

40 g of PFA was solved in 200 ml PBS and incubated at 60°C in the water-bath till PFA was completely solved. PH was set to 7.4. Aliquots were stored at –20°C, and they were further diluted with PBS before use.

Collagenase A stock

It was solved in PBS at a concentration of 100mg/ml. Aliquots were stored at –20°C.

DNAse I stock:

It was solved in PBS at a concentration of 10mg/ml. Aliquots were stored at –20°C.

CFSE stock:

5,6-Carboxy-Succinimidyl-Fluoresceine-Ester (CFSE) was kept as a stock solution (5mM in DMSO) and stored at –20°C.

Cell culture media

RPMI 1640 with HEPES, 10% FCS, 2mM Glutamine, 100 IU/ml Penicillin, 100µg/ml Streptomycin, 50µM 2-Mercaptoethanol was prepared. It was stored at 4°C.

0.005 M 2-Mercaptoethanol in PBS

178µl of 14.3 M 2-Mercaptoethanol was added to 500ml PBS. Solution was kept sterile and stored at 4°C. It was further diluted 1:100 in media.

6.1.4 Antibodies

Antigen	Isotype	Clone	Notes	Source
CD4	IgG2b κ, Rat	GK1.5		BD
CD8α	IgG2a κ, Rat	53-6.7	= Ly-2	BD
CD11b	IgG2b κ, Rat	M1/70	= Integrin αM, Mac-1 chain	BD
CD11c	IgG1, hamster	HL3	= Integrin αX chain	BD
CD16/32	IgG2b κ, Rat	2.4G2	= Anti-FCγR III + II / unconjugated	hybridome
CD25	IgG2b κ, Rat	3C7	= IL-2-Rezeptor-alpha chain	BD
CD40	IgG2a κ, Rat	3/23		BD
CD44	IgG2b κ, Rat	IM7	= Pgp-1, Ly-24	BD
CD49d	IgM κ, Rat			BD
CD62L	IgG2a κ, Rat	MEL-14	= L-selectin, LECAM-1, Ly-22	BD
CD69	IgG1, Hamster	H1.2F3		BD
CD80	IgG2, Hamster	16-10A1	= B7.1	BD
CD86	IgG2a κ, Rat	GL1	= B7.2	BD
F4/80	IgG2b, Rat	F4/80		BD
MHC II	IgG2a κ, Rat	2G9	I-Ad / I-Ed / bind also H-2b	BD
MHC II	IgG2a κ, Mouse	KH74	I-Ab / bind also I-Af, r, s, + v	BD
Vα2 TCR	IgG2a λ, Rat	B20.1		BD
Vβ5 TCR	IgG1 κ, Mouse	MR9-4		BD
CD206	IgG2a, rat	MR5D3	Mannose receptor	Serotec
		SF1	Bm1 screening	hybridome
BODYPY	IgG, rabbit		Anti-BODYPY-FL – DQ quenching	Mol. Probes

The used antibodies were either biotinilated or conjugated with different fluorochrome (FITC, PE, PerCP-Cy5.5, Pe-Cy7 or APC)

Streptavidin conjugated secondary antibodies:

FITC	BD (Heidelberg);
PE	BD (Heidelberg)
APC	BD (Heidelberg)
PerCPCy5.5	BD (Heidelberg)
Pe-Cy7	BD (Heidelberg)
Anti-rabbit-Alexa647	Molecular Probes (Netherlands)

- 7-Aminoactinomycin D (7-AAD) ($C_{62}H_{87}N_{13}O_{16}$, MW = 1270,45) (Sigma, Taufkirchen)
- PI – propidium iodide (Molecular probe, Netherlands)
- Hoechst 33342 (Sigma, Taufkirchen)
- Annexin V – bio BD (BD, Heidelberg)

Magnetic beads:

- α-Murine CD8-, CD11c- MicroBeads (Miltenyi, Bergisch Gladbach)

6.2 Mouse lines

1. Bm1: C57BL/6 mice bearing the mutant K^b molecule was provided by Dr. W.R. Heath, Melbourne, Australia.
2. C57BL/6: MHC Haplotype H-2K^b were bred at local animal facility (HET, University of Bonn)
3. OT-I mice: Vα2Vβ5 TCR recognises SIINFEKL in the context of H-2K^b. Dr. W.R. Heath, Melbourne, Australia, provided OT-I mice, on Rag-1-/- background.
4. $RAG2^{-/-}$: Dr. Frank Tache provided these mice, on a C57BL/6 background. (Aachen)
5. MR-/-: MR-/- mice on a C57BL/6 background were generated and provided by Dr. Michel C. Nussenzweig
6. CCR7-/-: mice on a C57BL/6 background were generated and provided by Prof. Dr. Martin Lipp.

For all experiments, mice between 8 and 16 weeks of age bred under SPF conditions were used in accordance with local animal experimentation guidelines.

6.3 Methods

Injection of soluble tracers

Tracers were diluted in PBS and injected intravenously in total volume 400µl. Animals were scarified after different time points.

Flow Cytometry

In general, approximately 10^6 cells were washed with 2 ml FACS buffer, centrifuged and the supernatant discarded. 20 µl of FACS buffer containing the appropriate primary monoclonal antibodies were then added to the cells and the cells were incubated for 30 minutes on ice. The cells were then washed in 2 ml of FACS buffer and where necessary, 20 µl of the secondary antibody was added and the cells were incubated for a further 30 minutes on ice. After an additional washing step, the cells were resuspended in 500µl FACS buffer and stored on ice.

Dead cells were excluded with either PI (0.5 µg/ml), 7AAD (0.5µg/ml) or by using Hoechst-33342 dye. Flow cytometry was performed on an LSR II (BD) or on FACS-CANTO (BD). Doublets were excluded using FCS-w/SSC-a gate. Data were analyzed using Flow-Jo software (Tristar, Ashland, OR), including calculation of division indices, which indicate the average number of cell divisions.

Removal of red cells

Single cell suspensions were depleted of red blood cells by centrifuging and resuspending in 5-10 ml RCRB for 1 minute. Cells were then centrifuged and resuspended in the appropriate medium.

Live cell counts

50 µl was taken from a single cell suspension of known volume and was mixed, by pipetting, with 50 µl of Trypan Blue Solution. 50 µl of the resulting suspension was then mixed, by pipetting, with 50 µl of a 4 % acetic acid solution to kill red blood cells. Live cells (Trypan Blue excluding cells) were then counted under a light microscope using a Neubauer chamber.

Centrifugation

Lymph node, bone marrow, spleen or kidney cell suspensions were centrifuged in 14 ml or 50 ml tubes for 5 minutes at 450 g at 4°C. 5 ml polystyrene tubes (flow cytometry) were centrifuged for 5 minutes at 200 g at 4°C. 96-well plates were centrifuged for 3 minutes at 200 g at 4°C.

Intracellular cytokine staining

Single cell suspension was prepared and cells were restimulated for 5 hrs in the presence of GolgiPlug (BD Bioscience Heidelberg Germany). Cells were fixed for 15 min at 4°C with 2 % Paraformaldehyde in PBS. Than washed and incubated for 20 min in PBS 0.1% BSA, 0.5 % saponin, which was followed by additional 30 min of incubation with the fluorochrome-conjugated antibody in PBS 0.1 % BSA, 0.5 % saponin and unconjugated rat IgG.

Annexin V staining

Cells were washed with PBS and 10^6 cells were resuspended in 100µl 1x binding buffer (BD, Heidelberg) and added 5 µl Annexin V-bio/sample (BD, Heidelberg). Cells were incubated at room temperature for 15 minutes followed by washing with binding buffer. They were further

incubated with SA-Fitc (BD, Heidelberg) together with 7AAD for 15 more minutes. After washing with binding buffer, samples were analysed immediately by flow cytometry.

Histology

Cyrosections from kidney were air dried and fixed with 4 % PFA, followed by blocking with PBS containing 3 % BSA for 2 hrs. Samples were stained with anti-BODYPY antibody 1: 300 in PBS 3% BSA for overnight at 4 °C, than were washed 3 times with PBS. As secondary antibody anti-rabbit Alexa-647 was used for 30 minutes at room temperature. After several washing step for nuclear staining Hoechst-33526 dye was used.

FITC conjugation

The 100mg/ml OVA concentration was used to label with FITC. 25 µl of carb/bicarb buffer, pH 9.2 was then added for every 0.5 ml of protein solution. Fluorescein-5-isothiocyanate (FITC) (Sigma. Taufkirchen) was weighed, dissolved in DMSO to give 1.5 mg/ml and immediately added to the antibody. The tube was covered in foil and gently rotated at room temperature for 30-40 minutes. A Sephadex G-25 column (Pharmacia Biotech, Uppsala, Sweden) was then washed with several ml of PBS and loaded with the FITC-conjugated OVA. The column was eluted with 2.5 ml PBS and the first band containing the FITC-conjugated OVA was collected.

Preparation of fluorescent soluble and cell-associated OVA

Soluble OVA was conjugated to a fluorochrome using an Alexa647 labelling kit (Invitrogen, Karlsruhe, Germany). Labeling procedure was carried out according to the manufacturer's guidelines. The labeling procedure involved gel-filtration as a final step for removal of low molecular weight molecules such as unbound fluorochrome. For cell-associated OVA,

splenocytes from Bm1 mice (2.10^8 cells/ml) were incubated with 10 mg/ml OVA-FITC for 10 min at 37°C, UV-irradiated with 15 mJ for 5 min and washed extensively.

Cross-presentation assay *in vivo*

OT-I cells were isolated from OT-I $Rag^{-/-}$ mice as described before (25). OT-I mice producing OVA-specific CD8 + T cells were crossed to a RAG-l-/- background to prevent endogenous rearrangement of TCR and to avoid generation of CD4+ T cells and B cells. Spleen and lymph nodes were collected and single cell suspension was prepared. To remove erythrocytes erythrolysis buffer (146mM NH_4Cl, 10mM $NaHCO_3$, 2mM EDTA) was used. Cells were labeled with 5- (and -6) carboxyfluorescein diacetate, succinimidyl (CFSE, Invitrogen, Karlsruhe, Germany) described before (183). Briefly 10-20 x 10^6 cells/ml were resuspend in PBS (Phosphate buffered saline) containing 0.1 % BSA (Bovine serum albumin type H1 GERBU, Germany), 5µM CFSE and stained at 37 °C for 10 minutes. Cells were washed with PBS; debris and aggregates were removed by filtering the cell suspension through a 70 µm mesh. Before adoptive transfer OT-I cell preparations were analyzed by flow cytometry. Suspension usually contained 70-85 % OT-I transgenic CD8 + T cells. $2x10^6$ OT I cells were injected intravenously into recipient mice.

For priming mice with soluble protein, OVA (grade V) was dissolved in PBS, to avoid peptide contamination gel filtration was performed using Sephadex G-25 (PD10 column, Amersham) according to manufacturer's protocol. Protein concentration was determined using BCA protein assay (Pierce, Bonn, Germany). 10µg/mouse g of soluble OVA was injected in 400µl PBS intravenously. An emulsion of 200µg OVA and 100µl CFA (Sigma Aldrich) was injected *s.c* into the flank of the mice.

Cross-presentation assay *in vitro*

20 minutes after injection of 1.5mg OVA, DCs were isolated from spleen and kidney. OT-I cells were isolated from OT-I rag-/- mice and further purified by a microbead- based CD8 T

cell isolation kit (Miltenyi). Purity was typically higher than 96 % of viable cells; contaminating CD11c+ cells were typically rarer than 0.2 %, NK1.1+ cells rarer than 0.03 %, and CD4+ cells were below 1 %. 5 x 10^4 DC were co-cultured with 2 x 10^5 CFSE labelled T cells and proliferation was assayed 48-72 hrs later by flow cytometry.

Isolation of DC from experimental animals

Cells were isolated from spleen and lymph nodes with digestion with Collagenase A (Roche Diagnostic, Mannheim Germany) and DNAse-I (Sigma Aldrich, Steinheim, Germany). For the preparations from the bone marrow, cells were collected after flushing through the femurs and tibias with PBS. Kidney was digested for 1 hrs at 37 °C using Collagenase A and DNAse-I and single cells suspension was let in MACS buffer at 4°C for 10 minutes to settle down tubular compartments, the upper 3/5 part of this cell suspension was further used either for staining or for CD11c MACS separation. $CD11c^+$ cells were in some cases enriched by magnetic separation using LS25 or MS25 columns (Miltenyi). Purity was typically higher than 85 %.

***In vivo* cytotoxicity assay**

In vivo cytotoxicity assays were performed as described (184). In brief, spleen cells were either pulsed with SIINFEKL (1 µg/mL, 45 min at 37°C) and labeled with a high concentration of CFSE (1 µM, 15 min at 37°C; CFSE high cells) or were mock treated and labeled with a low concentration of CFSE (0.1 µM; CFSE low cells). Cells were washed twice with PBS and equal numbers of cells from each population were injected *i.v.* (1x10^7 target cells). Animals were killed 4 h later and the presence of target cells in spleen and lymph nodes was analyzed by flow cytometry. To calculate specific lyses the following formula was used:
% specific cytotoxicity = $100 - (100 \times (CFSE^{high} / CFSE^{low})^{primed} / (CFSE^{high} / CFSE^{low})^{control})$.

Nephrectomy

Mice were anaesthetised (Ketamin (Pharmacia GmbH, Karlsruhe, Germany) 0.1 mg/g bodyweight and Rompun (aniMedica GmbH, Senden-Boesensell, Germany) 2 % 0.01 mg/g bodyweight). Nephrectomy was carried out as described before (25). Briefly, the abdominal cavity is entered from the basolateral side; the kidney is freed from surrounding tissue and is pulled out of the incision gently. The adrenal gland, which attached loosely to the anterior pole of the kidney is gently freed and returned to the abdominal cavity. The renal blood vessels and the urether are cauterized and the kidney is removed.

Transfer experiment

C57Bl/6 mice were injected with 2 million CFSE labeled OT I cells. Next day they were primed with OVA (10µg/g) *i.v.* or with OVA-CFA *s.c.*. On day 2 total renal LN cells were pooled from 6-8 animals, and together with the cLN cells from OVA-CFA primed animal aliquot was taken for analysis of TCR and CD8 expression. Than cells were transferred into $Rag^{-/-}$ recipients and 12 days later LN and spleen was collected from the $Rag^{-/-}$ mice and analyzed for TCR and CD8 expression.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Comparisons were drawn using a two-tailed Student's t-test (Prism 4, Graphpad Software Inc., San Diego CA USA).

7. Results

7.1 Part 1: The mannose receptor mediates uptake of soluble, but not of cell-associated antigen for cross-presentation

7.1.1 Dendritic cells in the secondary lymphoid organs express MR *in vivo*

The role of the MR in antigen uptake and presentation by DCs was proposed based on studies in human dendritic cells *in vitro* (95, 121). Recently it has been also conformed in the lab of Prof. Kurts in murine bone marrow derived DCs (182). To elucidate the role of MR in antigen presentation *in vivo*, first the expression of MR was determined in secondary lymphatics by flow cytometry. Using conventional surface staining, only weak MR expression was detected on DCs isolated from spleen, BM or cLN. However, intracellular staining revealed the presence of the MR in all organs tested (Fig. 6).

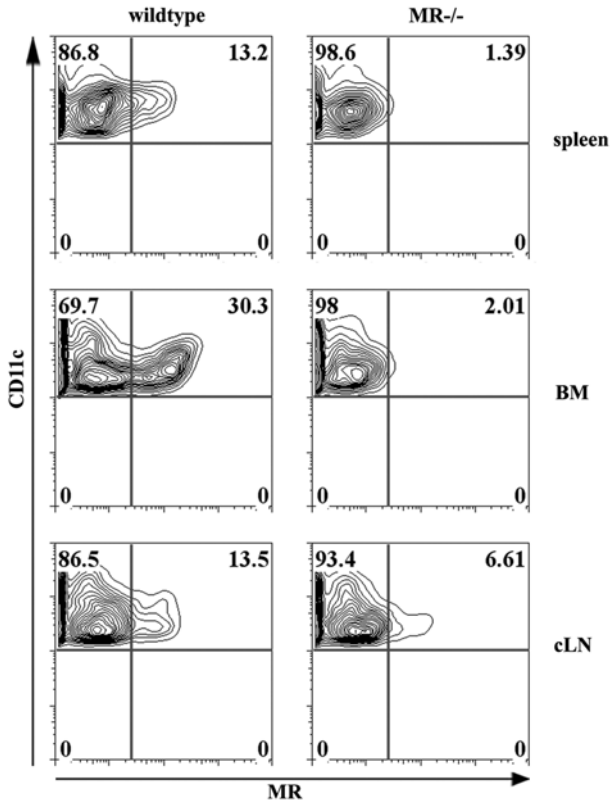

Figure 6. MR expression in different secondary lymphoid organ
DC from spleen, BM, cLN were isolated from C57Bl/6 and MR deficient mice and subsequently stained for CD11c and MR. For MR cells were stained intracellularly. Shown are the viable $CD11c^+$ cells present in the different organs.

7.1.2 Uptake of soluble antigen by DC *in vivo* is mediated predominantly via the MR

To clarify the *in vivo* handling of soluble molecules in DC possibly mediated by MR, ovalbumin (OVA) as a model antigen was used.

After injection of fluorochrome-labeled soluble OVA into C57Bl/6 mice, DCs were isolated from the spleen. The uptake of soluble OVA by these cells showed close correlation with the expression of the MR (Fig. 7). A small proportion of captured OVA however was not in MR^+ DCs indicating that this molecule was taken up by DCs via an MR-independent mechanism.

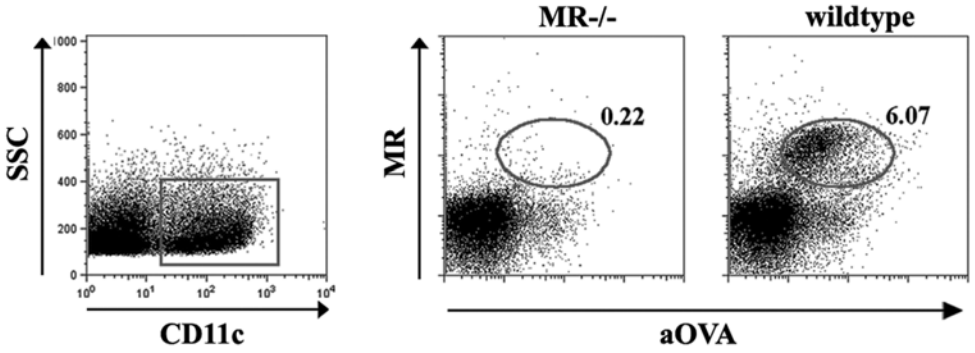

Figure 7. Uptake of soluble OVA *in vivo* is mediated predominantly via the MR
C57Bl/6 mice were *i.v.* injected with aOVA (5µg/ body weight). After 45 minutes, spleen was digested with Collagenase and DNAse-I containing medium. The resulted single cell suspension from the spleen was stained for surface expression of CD11c and for intracellular expression of the MR.

Consistent with above finding, systemic injection of Alexa-647 labeled OVA (aOVA) into MR-/- mice showed significant but incomplete reduction of uptake in DCs isolated from spleen, cLN and bone marrow (Fig. 8 A, B). The decline was most evident in the spleen and in the bone marrow. In the cLN, only moderate endocytosis of aOVA could be observed, which was further reduced in MR-/- mice, albeit not significantly (Fig 8 A, B).

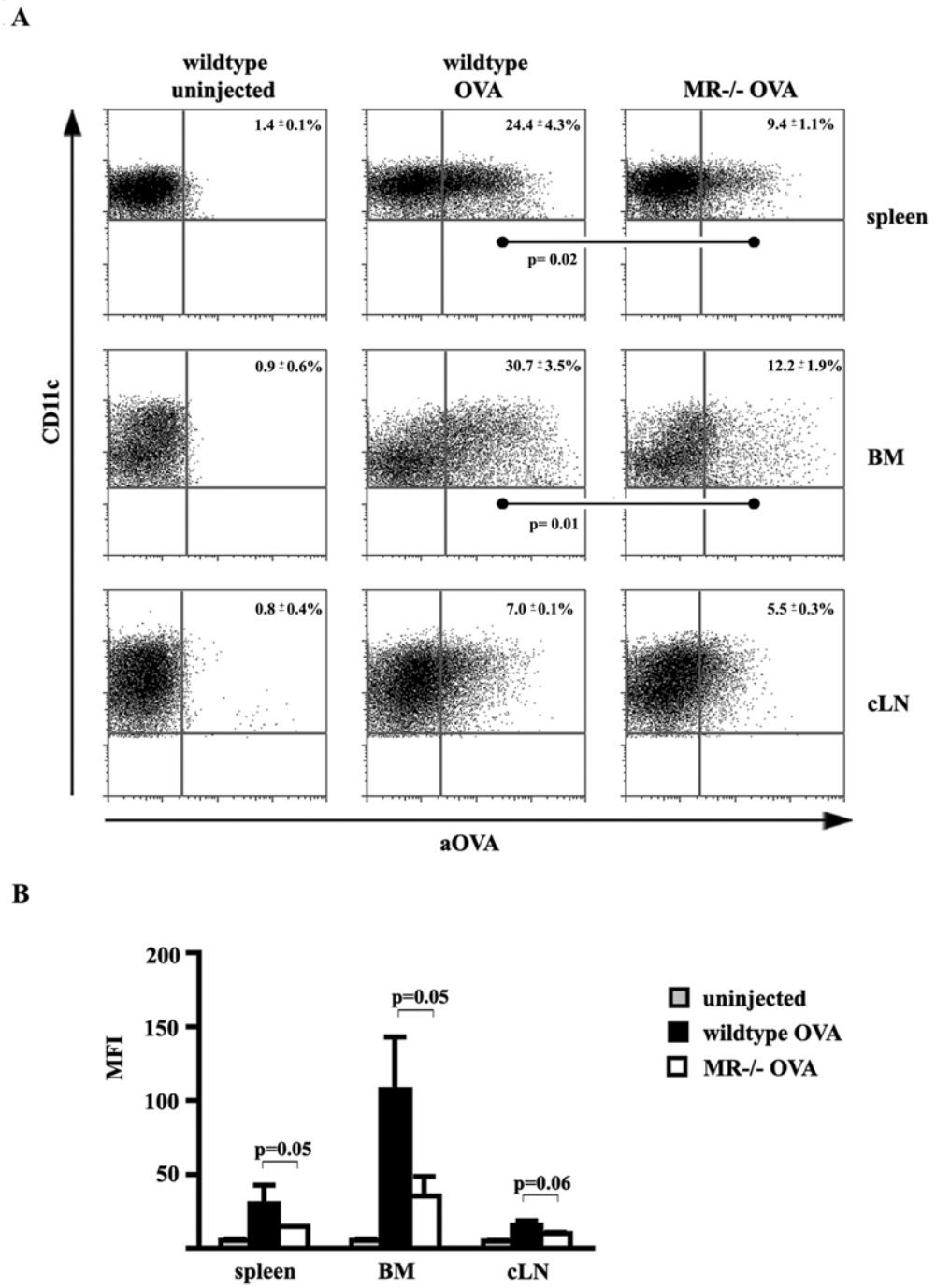

Figure 8. Uptake of soluble OVA *in vivo* is mediated predominantly via the MR

A Wildtype and MR deficient mice were *i.v.* injected with aOVA (5µg/body weight). After 45 min, DC were isolated from the spleen, BM, and cLN and analyzed by flow cytometry. Numbers give the proportions of $aOVA^+$ cells ± SD within the viable $CD11c^+$ cells. (n=3 or 4 mice)

B MFI ± SD of the viable $CD11c^+$ cells from B.

To rule out that the above reduction of OVA uptake was due to changes of DC subpopulations present in MR-/- mice, the percentage of $CD8\alpha^+$ lymphoid and $CD11b^+$ myeloid DC were determined in the different organs. Both DC subtypes in MR deficient mice compared to wildtype were unaltered. The percentages ± SD is summarized in table 1.

	$CD8\alpha^+$	**$CD11b^+$**
BM wildtype / MR-/-	6.25 ± 1.2 / 6.5 ± 0.9	32.6 ± 0.2 / 29.25 ± 3.6
Spleen wildtype / MR-/-	26.55 ± 0.4 / 25.5 ± 1.9	42.8 ± 0.8 / 43.9 ± 1.1
cLN wildtype / MR-/-	19.45 ± 0.2 / 19.2 ± 1.4	35.1 ± 2.9 / 31.65 ± 0.5

Table 1. Percentage of $CD8\alpha^+$, $CD11b^+$ DCs in wildtype and MR-/- mice
DCs were isolated from wildtype and MR-/- animals from spleen, cLN, and bone marrow and stained for CD11c, CD11b and CD8. Percentages are given ± SD (n=3 animals/group)

These findings demonstrated that the MR was involved also in the *in vivo* uptake of soluble OVA, but it was not essential, as opposed to its role *in vitro* (182). Indeed, some DC had taken up OVA but did not express MR. The mechanisms that partially compensated for the absence of MR *in vivo* remain to be identified

7.1.3 MR is dispensable for *in vivo* uptake of cell-associated antigen

In order to compare the fate of soluble and cell-associated antigens *in vivo* intravenously injected bm1 splenocytes were used to carry the antigen to DCs located in the different organs. For the uptake experiments bm1 splenocytes were loaded with FITC-labeled OVA. In order to demonstrate efficient antigen delivery into these cells, loading with FITC-labeled OVA was performed at 4 °C and at 37°C, since intracellular uptake is energy dependent as opposed to extracellular coating. Indeed, uptake at 37°C was significantly higher than at 4°C, suggesting that some of the antigen was actively endocytosed by these splenocytes (Fig 9). After OVA-

coating apoptosis was induced by UV irradiation followed by immediate injection of these cells into recipient mice (about 30 % of the cells were Annexin-V+ and PI-, apoptotic).

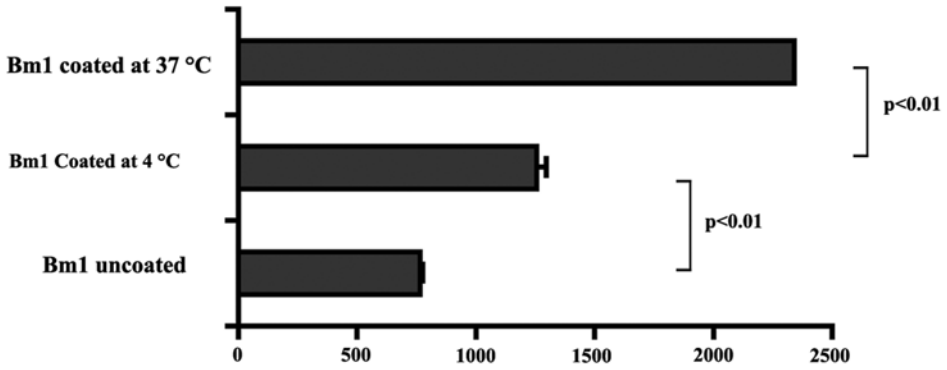

Figure 9. Bm1 splenocytes were loaded with Fitc-OVA
Bm1 splenocytes were incubated with Fitc-OVA for 10 min at 37°C and 4°C and than analyzed for uptake of fluorescent soluble antigen by flow cytometry.

UV-irradiated bm1 splenocytes loaded with FITC-OVA were injected *i.v.* into MR deficient and wildtype mice. DCs from the spleen and from the bone marrow of C57Bl/6 mice showed significant, and the cLN only marginal uptake of cell-associated antigen, consistent with reports by others (Fig. 10 A, B) (40, 55). However in MR deficient mice, uptake was unaltered, implying that cell-associated antigen had been internalized by mechanisms other than MR-mediated endocytosis.

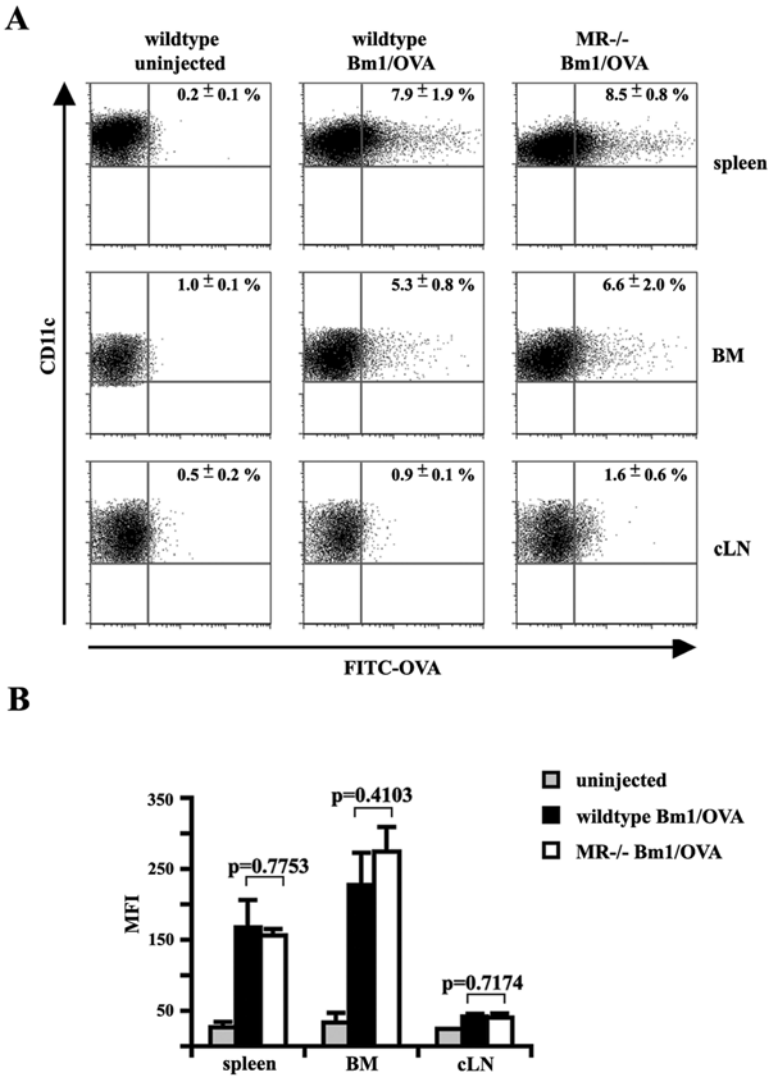

Figure 10. MR is dispensable for *in vivo* uptake of cell-associated antigen
A Fitc-OVA loaded Bm1 cells (20 x 10^6) were UV irradiated and injected into wildtype and MR deficient mice. 1hr later, DCs were isolated from spleen, BM and cLN, and the proportion of $aOVA^+$ cells in the viable $CD11c^+$ cells was determined. (n=3 or 4 mice)
B MFI ± SD of the cells from A.

7.1.4 The MR contributes to *in vivo* activation of $CD8^+$ T cell by cross-presentation of soluble but not of cell-associated antigen

To assess the *in vivo* role of the MR for CD8 T cell activation by cross-presentation, CFSE-labeled OT-I transgenic T cells were injected into wildtype and MR-deficient mice. Two days after priming with soluble OVA, the proliferation of OT I cells in spleen, bone marrow, and cLN were analyzed. In MR-/- mice, proliferation of these T cells was significantly diminished, suggesting MR mediated endocytosis in DCs shuttle antigens for the cross-presentation pathway also *in vivo* (Fig. 11 A, B).

In order to demonstrate the cross-presentation of cell-associated OVA, antigen loaded and UV irradiated bm1 splenocytes were used. Bm1 mice bear a mutant H2-K^b protein that cannot present OVA to OT-I cells allowing the exclusion of direct presentation by antigen carrying cells, thus only the recipient DCs after capturing and processing the antigen containing splenocytes were able to induce CD8 T response, as reported by others before (36).

Proliferation of OT I cells in the spleen and in the bone marrow was not reduced in MR-/- mice in agreement with the equal uptake capacity of this antigen type in both mice (Fig. 10 A, B and Fig. 12 A, B). Furthermore, in the cLN no proliferation of OT I cells was observed in consistent with the marginal antigen uptake occurring in this location *in vivo* (Fig. 10 and 12 A, B). Thus, mechanisms other than MR-mediated antigen uptake were responsible for CD8 T cell activation by cross-presentation of cell-associated antigen.

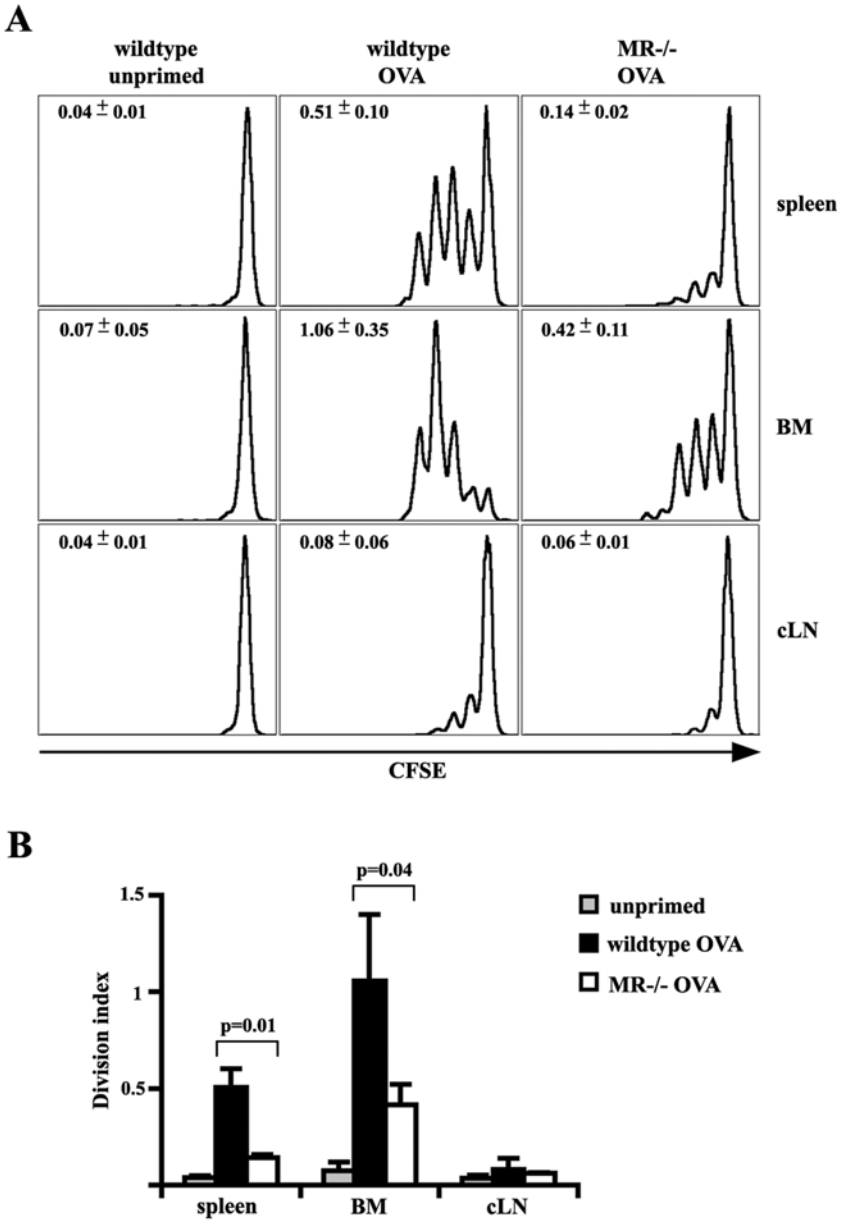

Figure 11. MR contribute to the *in vivo* activation of $CD8^+$ T cell by cross-presentation of soluble OVA

A CFSE-labeled OT I cells (2×10^6) were injected into wildtype and MR deficient mice. After 18 hrs, 5µg OVA /body weight was injected *i.v.* After an additional 40 hrs, the proliferation of OT-I cells was analyzed in the spleen, BM, and the cLN. Shown are the living $CFSE^+CD8^+$ cells present in the different organs.

B Division indexes of proliferating cells from experiment A.

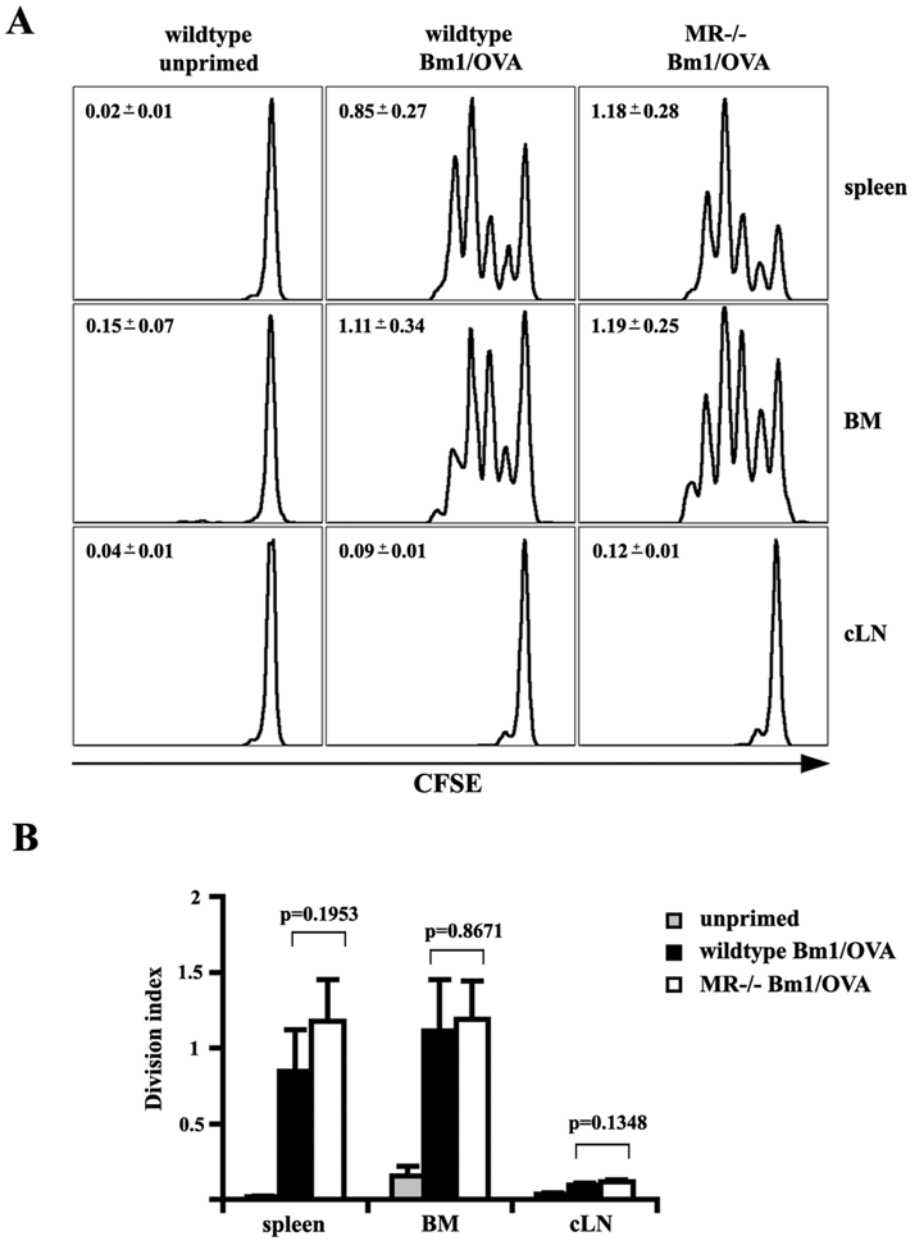

Figure 12. MR does not contribute to the *in vivo* activation of $CD8^+$ T cell by cross-presentation of cell-associated OVA

A CFSE-labeled OT I cells (2 x 10^6) were injected into wildtype and MR deficient mice. After 18 hrs, 20 x 10^6 OVA-loaded, UV irradiated splenocytes were injected *i.v.* After an additional 40 hrs, the proliferation of OT-I cells was analyzed in the spleen, BM, and the cLN. Shown are the living $CFSE^+CD8^+$ cells present in the different organs.

B Division indexes of proliferating cells from experiment A.

The next experiment was designed to ensure that the phenotype observed above in $CD8^+$ T cell proliferation was due only to the inability of DCs to efficiently capture soluble OVA in the absence of MR rather than other factors such as abolishment in recirculation of OT I cells activated elsewhere. First CD69 expression on OT-I cells was determined 15 hrs after priming. In case of cell-associated antigen no alteration was observed in the upregulation of CD69 on the activated T cells (Fig. 13 A). The percentage of $CD69^+$, activated OT I cells was significantly reduced in MR deficient mice after priming with soluble OVA, indicating a diminished $CD8^+$ T cell activation *in situ* (Fig. 13 A).

Moreover, the level of co-stimulatory molecules expressed on DC showed no difference between wildtype and MR deficient mice (Fig. 13 B), indicating that variations in costimulation could not explain the reduced activation of $CD8^+$ T cells in the presence of soluble OVA.

Taken together, the diminished proliferation of OT I cells was due to the decreased antigen uptake capacity of DC via the MR, thereby causing limited activation of antigen specific T cells at the priming site.

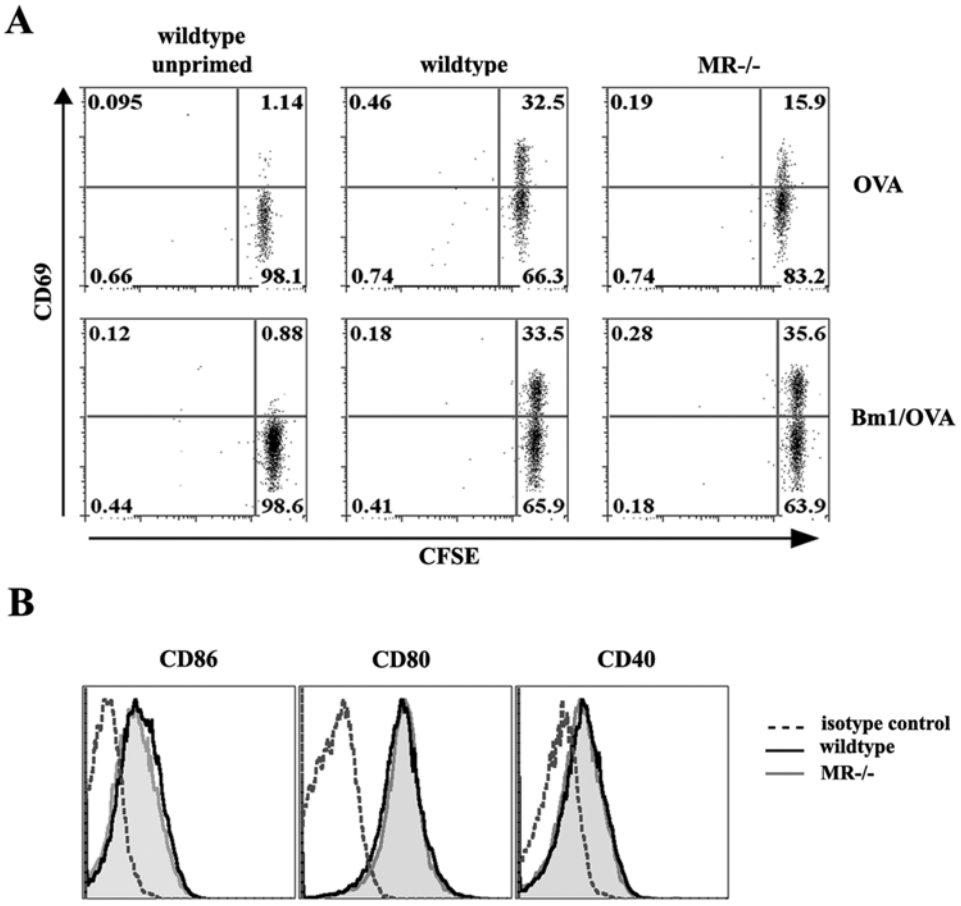

Figure 13. No effect of MR on the expression of CD69 on activated T cell and on expression of costimulatory molecule by DC

A CFSE-labeled OT I cells (2 x 10^6) were injected into wildtype and MR deficient mice. After 18 hrs, 20 x 10^6 OVA-loaded, UV irradiated splenocytes or 5µg OVA / body weight were injected *i.v.* After an additional 15 hrs, the CD69 expression of OT-I cells was analyzed in the spleen. Shown are the $CFSE^+CD8^+$ cells present in the spleen.

B DCs were isolated from wildtype and MR deficient mice and surface staining was carried out in order to analyze the expression of costimulatory molecules such as CD86, CD80, and CD40.

7.1.5 The MR and the cross-presenting APC in the spleen

It has been described that $CD8^+$ DC were responsible for the cross-presentation of both cell-associated and soluble antigen in the spleen (37, 39). Only $CD8^+$ DCs had been previously shown to capture apoptotic, dying cells from the circulation (40, 55). Indeed, MR deficient mice did not show alteration in $CD8^+$ DC mediated uptake of cell associated OVA (Fig. 10, 14 A). In case of soluble antigen however, both lymphoid and myeloid splenic DC were capable of endocytose soluble OVA from the blood in consistent with previous findings (39) (Fig. 14 B). MR deficient mice showed a reduction of aOVA in splenic DC (Fig. 8 A, B), which raised the question whether the reduction in the uptake was DC subtype specific. Indeed, expression of MR by freshly isolated splenic DC was exclusively restricted to the $CD8^+$ subset. Thereby expression of MR allows distinguishing between DCs that are capable of cross-presentation. Taken together, the expression of MR, besides CD8α (37) and Dec-205 (185), another absolute marker expressed exclusively by the cross-presenting APC *in vivo*.

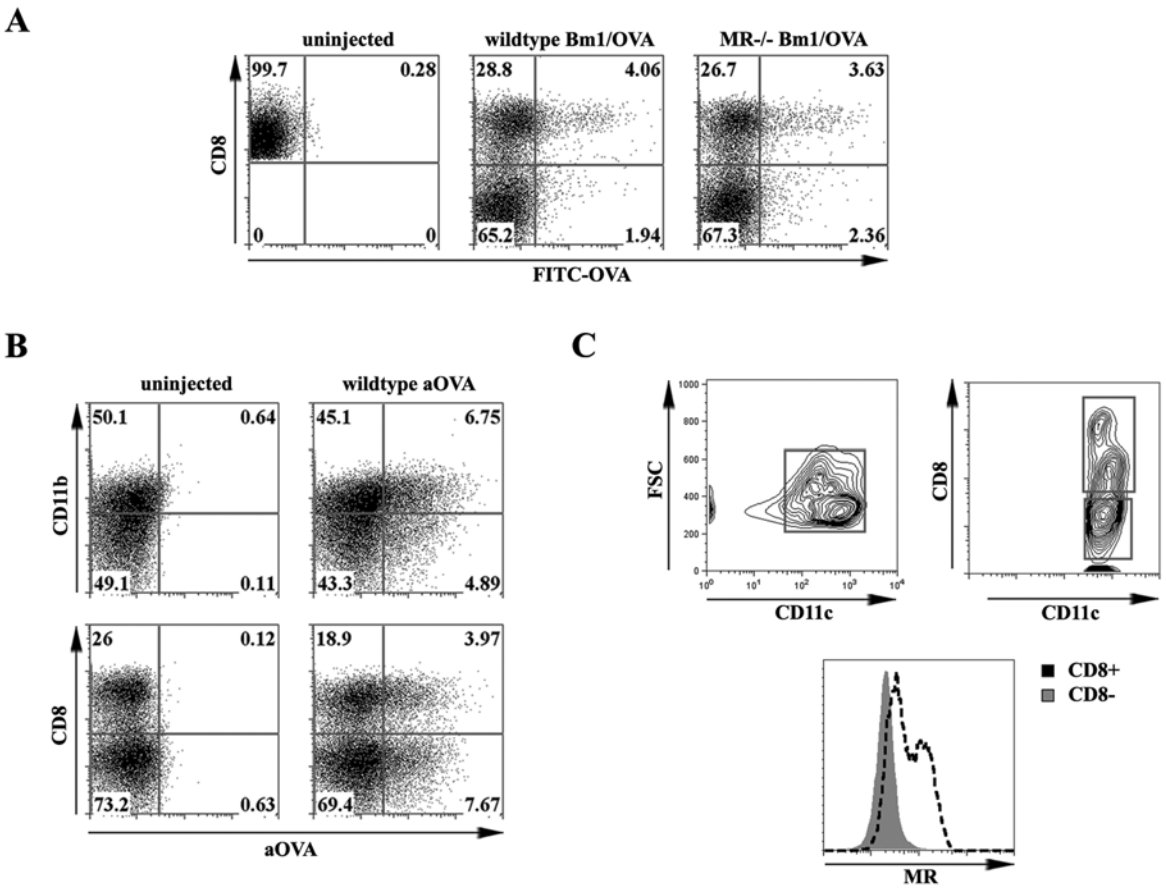

Figure 14. The MR and the cross-presenting DC in the spleen

A Wildtype and MR deficient mice were injected *i.v* with 20 x 10^6 Fitc-OVA loaded and UV irradiated splenocytes. 1 hr later DC from spleen were prepared and analyzed for CD8 expression. Shown are viable $CD11c^+$ cells.

B Wildtype mice were injected *i.v* with 5µg/ body weight aOVA. 45 min. later DC from spleen were prepared and analyzed for CD8, and CD11b expression. Shown are viable $CD11c^+$ cells.

C DC were isolated from wildtype mice and stained for MR and CD8. Shown is the viable $CD11c^+$ cells gated on $CD8^-$ and $CD8^+$ after doublet exclusion.

7.2 Part 2: Lymph node-resident dendritic cells induce T cell cross-tolerance against tissue-derived antigen

7.2.1 Kidney dendritic cells preferentially endocytose filtrated antigens

Molecules below albumin molecular size (68 kDa) pass the kidney glomerular filter and are reabsorbed by tubular epithelial cells and kidney dendritic cells (kDC) (23, 30, 31). This suggested that the concentration of the glomerular filtrate in the tubular system might cause enrichment of filtrated proteins in kDC. To test this hypothesis, $Alexa_{647}$-labeled OVA (aOVA) and 500 kDa FITC-labeled dextran were co-injected *i.v.* into C57Bl/6 mice. The large dextran served as marker for non-filterable molecules, which DC can acquire only from the blood. In contrast, aOVA should be available also from the tubular ultrafiltrate, because its molecular weight of 45 kDa renders it filterable. 20min after injection, DC from the spleen and from cutaneous LNs (cLNs) had internalized both aOVA and FITC-dextran (Fig. 15). Splenic DC endocytosed both molecules more efficiently, but the ratio between them was similar (note the lines in Fig. 15). In contrast to this, kDC efficiently endocytosed aOVA but little FITC-dextrane (Fig. 15) indicating superior uptake of filtrated molecules and inferior uptake of blood-borne molecules.

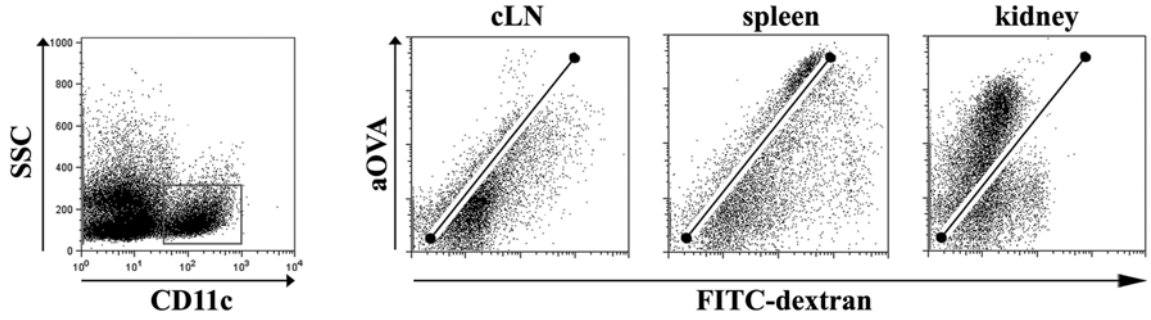

Figure 15. Selective uptake of filtrated antigen by kDC
C57Bl/6 mice were *i.v.* injected with 8µg/g body weight of $Alexa_{647}$-labeled OVA (aOVA) and of 500 kD FITC-dextran. After 20 min, $CD11c^+$ DC from the cutaneous LN (cLN), the kidney and the spleen were analyzed for fluorescence uptake. A line was added to the dot-plots to indicate the ratio between uptake of small and large molecules. Note that in kidney DC (kDC), this ratio was in favor of small molecules.

7.2.1.1 Enrichment of filtrated antigen in the kidney is remarkably fast

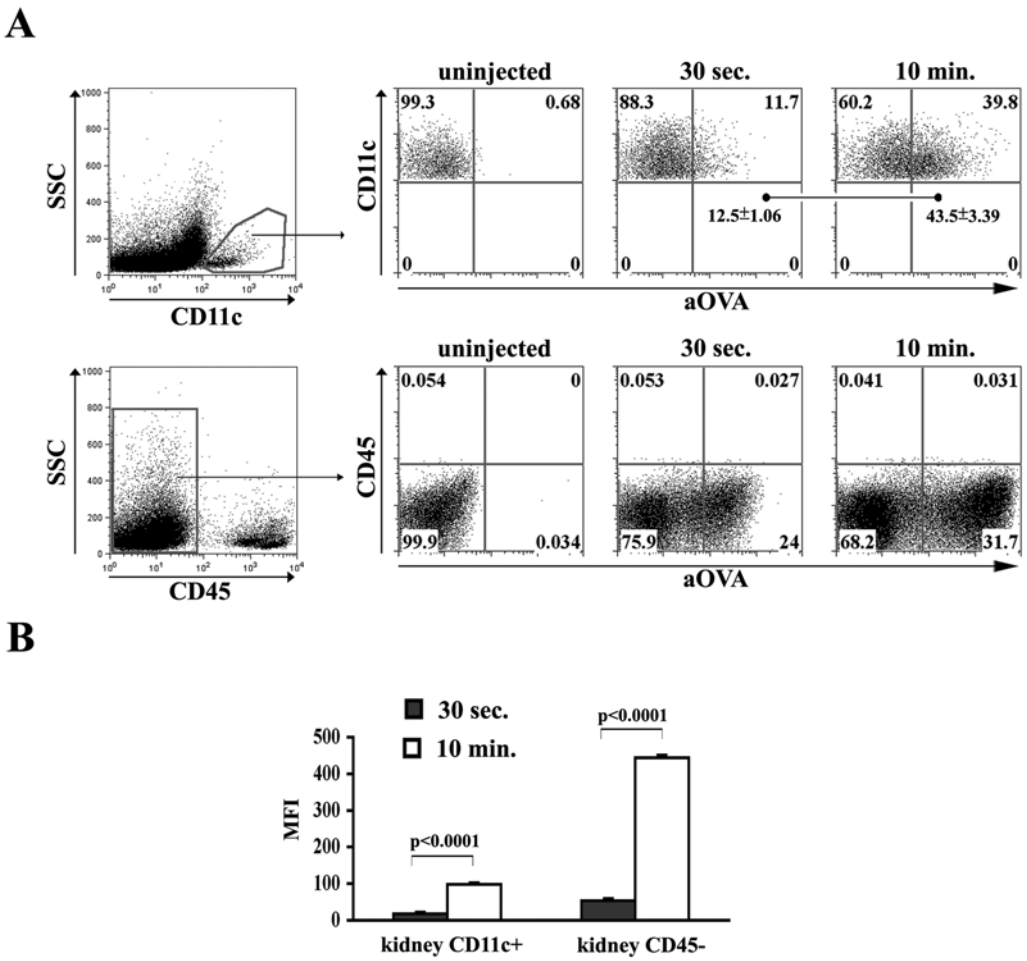

Figure 16. Rapid uptake of filtrated antigen by kDC

A Mice were *i.v.* injected with 8µg/g body weight aOVA. After 30 sec or 10 min, fluorescence uptake by $CD45^-$ intrinsic kidney cells, and $CD11c^+$ kDC was determined. Numbers in the lower right dot-plots give proportions of DC containing aOVA ± SD (n=3).

B Statistical analysis of the MFI of aOVA uptake in Fig. 16 A. Background MFI of cells from uninjected animals was subtracted (n=3).

The kidney DC acquired *i.v.* injected aOVA very rapidly. Already 30 sec after injection, about 12 % of the kDC had accumulated some aOVA (Fig. 16 A). After 10 min, the mean fluorescence intensity (MFI) had risen more than 5-fold (Fig. 16 A, B), and antigen was detectable within nearly half of the kDC, equivalent to 30–50x10^3 DC per kidney. Uptake of aOVA was even larger in kidney cells deficient for the common leukocyte marker CD45 (Fig.

16 B). Histology identified these cells upon their typical morphology as tubular epithelial cells, consistent with their established function of reabsorbing filtrated protein (Fig. 17.) (28).

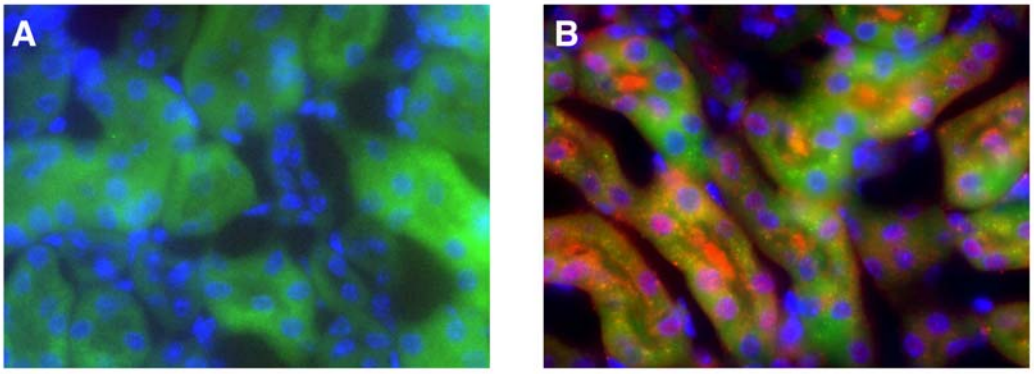

Figure 17. Rapid accumulation of filtrated antigen in tubular cells of the kidney
Mice were *i.v.* injected with 8µg/g body weigh of DQ-OVA. After 10 min, fluorescence uptake was determined by anti-BODIPY + anti-goat Alexa-647 antibody (red), nucleus was stained with Hoechst (blue), the high autofluorescence of the kidney shown in green. A shows uninjected, B injected animal.

7.2.1.2 DC of the myeloid phenotype endocytose filtrated antigens but do not induce T cell activation *ex vivo*

DC in the kidney that endocytosed filtrated molecules showed the $CD11b^+$ myeloid phenotype (Fig. 18 A), whereas $CD8\alpha^+$ lymphoid and $Gr\text{-}1^+$ plasmacytoid DC were absent (Fig. 18 A). Later is consequent with previous findings that these DC subtypes are completely absent in the normal kidney (21). Endocytosis of aOVA by kDC did not result in DC maturation as evidenced by lack of CD80 or MHC II upregulation (Fig. 18 B).

In vitro co-culture of antigen specific T cells (OT-I) with kidney DCs isolated from soluble OVA injected animal did not result in efficient T cell activation compared to splenic DC isolated from the same injected animal. (Fig. 19) Nevertheless, kDCs coated with SIINFEKL were able to induce T cell proliferation similar to splenic DCs, suggesting that the difference in the observed phenomenon is not dependent on the level of co-stimulation expressed by kDC.

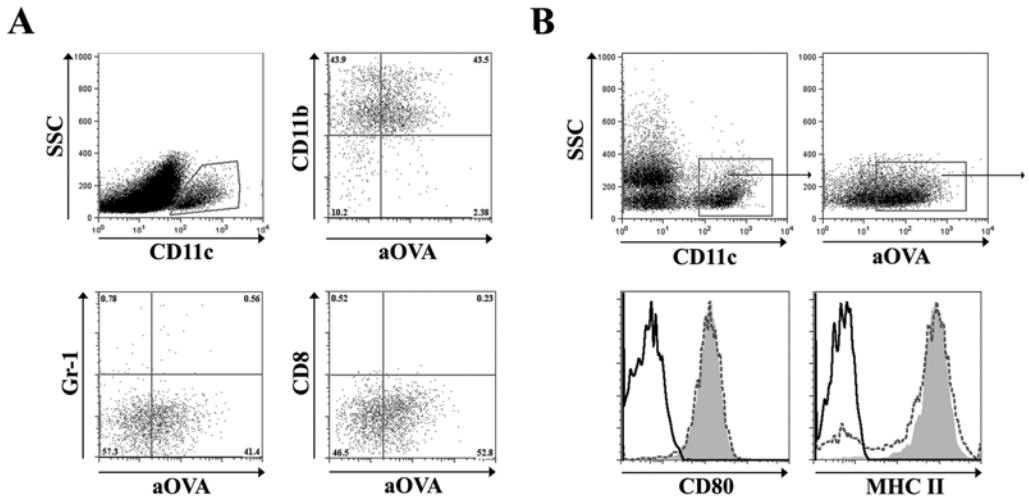

Figure 18. Phenotypic analysis of kDCs endocytosed filtrated molecules

A Expression of CD11b, CD8α and Gr-1 by $CD11c^+$ kDC. Dot-plots show results typical of 3 individual experiments.

B Expression of CD80 and MHC II by $CD11c^+$ kDC before (dashed line) and 1 h after aOVA injection aOVA (tinted). Isotype controls are shown as bold lines.

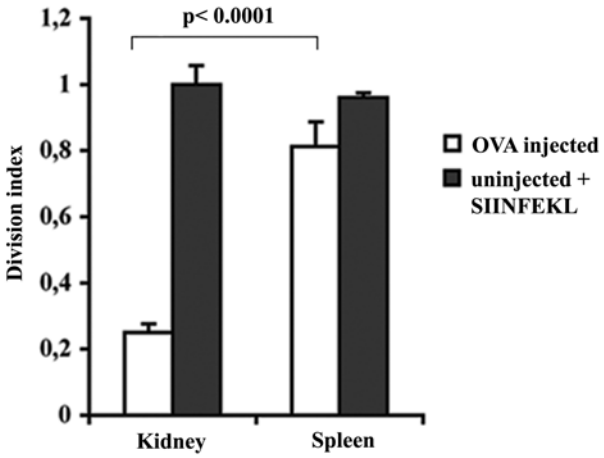

Figure 19. *In vitro* ability of kDC to induce CD8 T cell response

C57Bl/6 mice were injected with 1.5 mg OVA, 20 minutes later dendritic cells from kidney and spleen were isolated. $5x10^4$ DCs were co-cultured with $2x10^5$ CFSE labeled OT-I cells. Proliferation was measured 72 hrs later by FACS. As a positive control kidney and splenic DC from uninjected animals coated with SIINFEKL was used.

7.2.2 Rapid cell independent transport of filtrated antigen to rLN DC

7.2.2.1 Enrichment of filtrated molecules in rLNs elicited particularly fast kinetic and was dependent on filtration process occurring in the kidney

As has been reported before kidney autoantigens are presented to $CD8^+$ T cells in the rLN (11, 25). To test whether also antigen filtrated in the kidney reached this LN, mice were injected *i.v.* with aOVA and uptake by rLN cells was determined. In order to distinguish aOVA taken up from the circulation and aOVA arriving from the kidney, the rLNs were compared to a kidney non draining LN, which could acquire antigen only from the circulation such as the cLN. Indeed, more cells captured aOVA in rLNs than in cLNs (Fig. 20 A), and higher antigen uptake was detected as shown by the comparison of mean fluorescence intensity (MFI) of aOVA in $CD11c^+$ cells (Fig. 20 A, B), indicating the presence of excess kidney-derived filtrated aOVA in the rLNs. Importantly, the superior aOVA accumulation in the rLNs over the cLN was seen already 90 sec after aOVA injection and was further elevated after 5 min (Fig. 20 A, B). Additionally, this fast enrichment was dependent on the filtration process occurring in the kidney, since left nephrectomy (NX) before aOVA injection abrogated accumulation of filtrated antigen in the left rLN, while that in the right rLN remained unaffected (Fig. 20 C). At the site of the nephrectomy, rLN showed uptake comparable to that in cLN, indicating similar abilities for both LN to acquire antigens from the blood. Thus, aOVA accumulation in rLNs depended on its enrichment in the kidney, rather than on particularities of these LNs such as microcirculation or special localization close to major vessels.

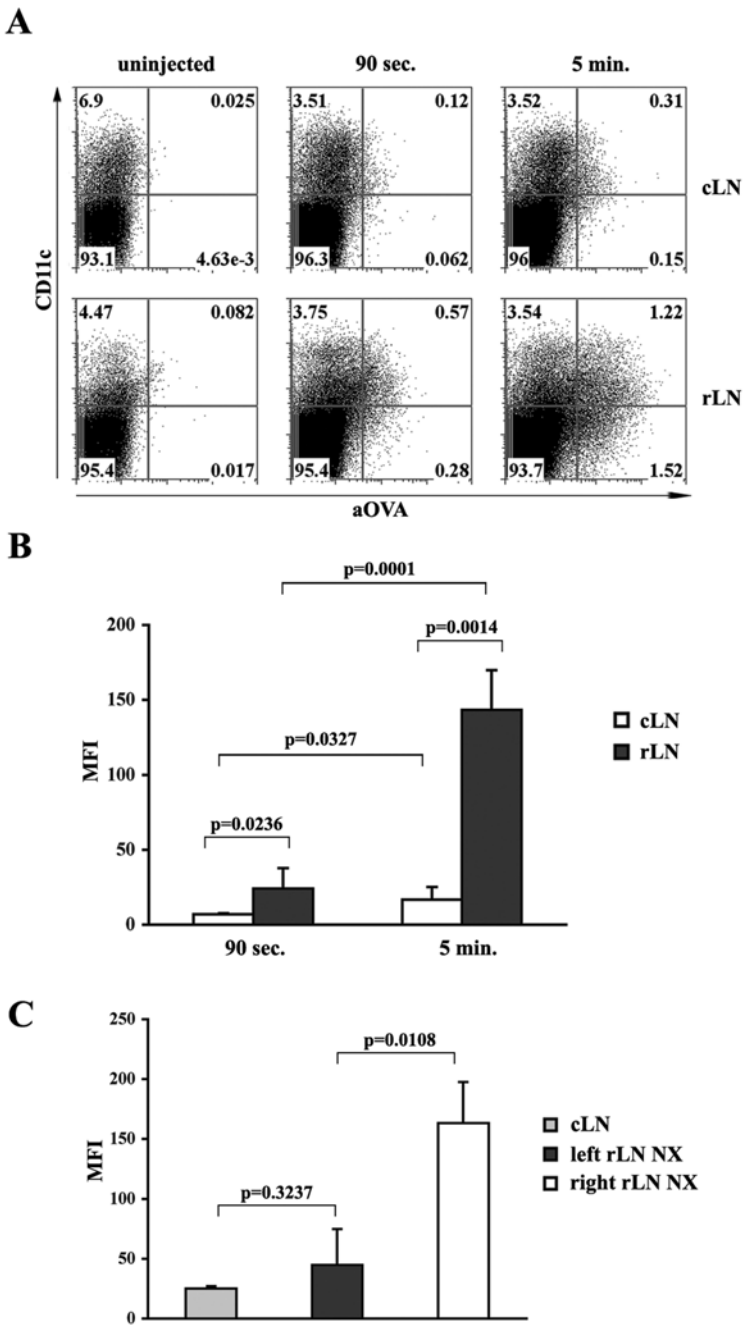

Figure 20. Rapid accumulation of filtrated antigen in rLN DC

A C57Bl/6 mice were injected *i.v.* with 8µg/g body weight aOVA. After 90 sec or 5 min, cells from rLNs and cLNs were stained for CD11c. Dot plots show typical aOVA content and CD11c expression in total viable $7AAD^-$ LN cells.

B Statistical analysis of Fig. 20 A, giving MFI of $CD11c^+$ DC 90 sec and 5 min after aOVA injection (n=5 mice).

C 8µg/g body weight aOVA was *i.v.* injected one day after surgical removal of the left kidney. aOVA content in $CD11c^+$ DC was detected in the left and right rLN and in the cLN (n=6 mice, rLNs from two animals were pooled for analysis).

7.2.2.1 Filtrated antigen accumulates predominantly in CD11c^{lo} and moreover reaches some CD8α^{+} DCs of the renal LN

Most rLN cells that contained filtrated aOVA were CD11c^{+} CD11b^{+} conventional myeloid DC, while CD11c^{-} CD11b^{+} macrophages represented a minority (Fig. 20 A, Fig. 21 A). The absolute number of aOVA^{+} DC was about 20-fold lower in one rLN than in one kidney (Fig. 21 B). Interestingly, in rLNs, aOVA was detected mostly in CD11c^{lo} DC (Fig. 20 A, 21 A), whereas aOVA^{+} DC in the kidney were predominantly CD11c^{hi} (Fig. 16 A). The rLN differed from the kidney also by containing some CD8α^{+} DC that had captured aOVA (Fig. 21 C).

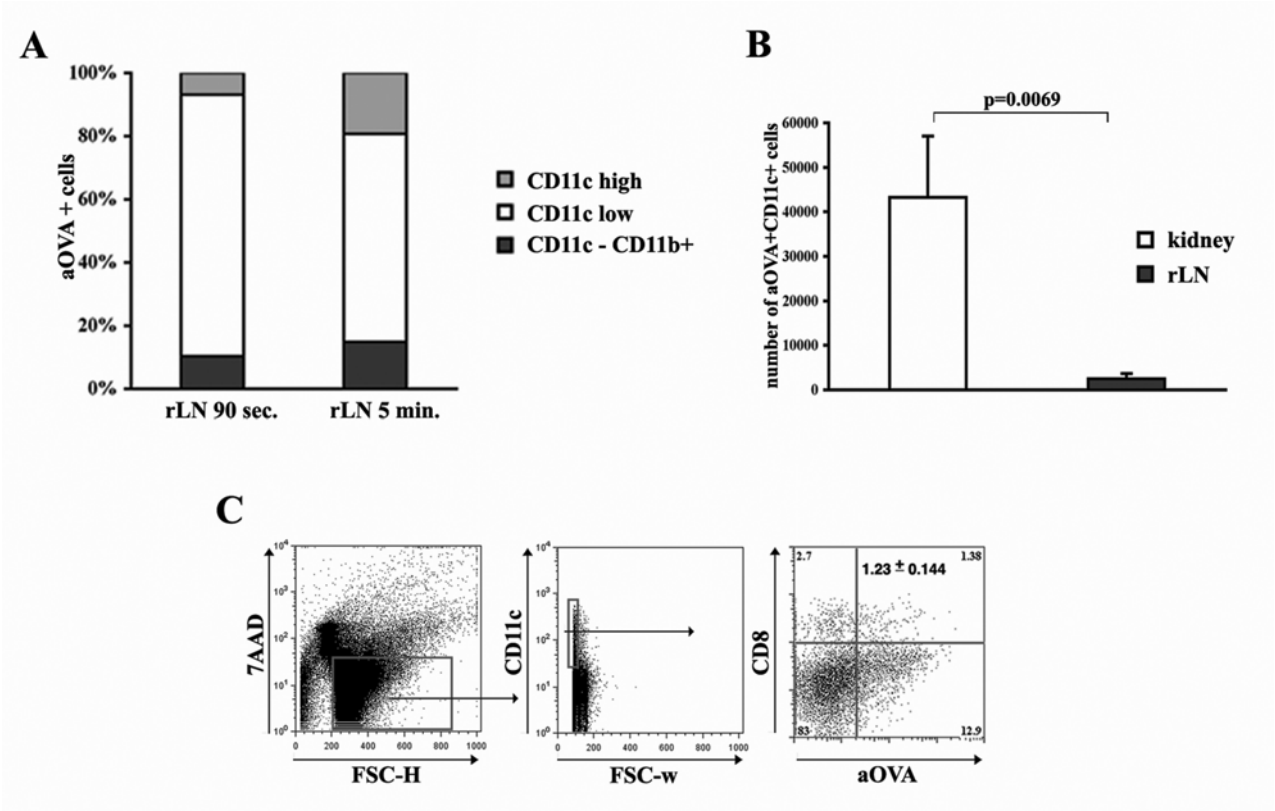

Figure 21. Phenotype of cells accumulating aOVA in the rLN

A Proportions of CD11c^{lo}, CD11c^{hi} DC and of CD11c^{-} CD11b^{+} cells within the aOVA-containing rLN cells from Fig. 20 A.

B Absolute numbers of CD11c^{+} aOVA^{+} DC in one kidney and in the left rLN (n=3 mice)

C Viable CD11c^{+} DCs in rLNs were analyzed for CD8α expression and aOVA uptake. Doublet events were excluded by FSC-w gate. The number gives mean ± SD of CD11c^{+} positive for both parameters (n=3). Without doublet exclusion, this value was 2,8 ± 0.5 %.

7.2.2.2 Antigen uptake in rLNs depends on the MR, but not on CCR7

These phenotypic differences between aOVA^{+}DC in the kidney and renal LN and the rapid kinetics of aOVA accumulation suggested cell-independent antigen transport into rLNs. To test this hypothesis, recent discovery was used that endocytosis of soluble OVA by DC was facilitated by the mannose receptor (MR) (Fig. 8 A, B). As expected, this receptor was used by rLN DC for uptake of filtrated aOVA, as evidenced by lack of this uptake in MR$^{-/-}$mice 5 min after injection. (Fig. 22 A). In contrast, kDC had internalized somewhat less aOVA at this time (Fig. 22 A), and this occurred in a MR-independent fashion in consistent with that MR expression was not detectable by kDCs (Fig. 22 B) The absence of DC containing high amounts of MR-endocytosed aOVA from the kidney argued against this organ as origin of such DC in rLNs. Suggesting LN-resident DCs have taken up and accumulated filtrated antigens arriving from the kidney presumably via the lymphatics in a cell independent manner. To confirm this interpretation, uptake of OVA was investigated in CCR7$^{-/-}$ mice, in which DC migration from non-lymphoid organs to their draining LN is incapacitated (156). Despite this defect, rLN DC of CCR7$^{-/-}$ mice showed unimpaired accumulation of DQ-OVA (Fig. 22 C), an alternative tracer for filtrated antigen. However the percentage of CD11c^{+} cells was lower in rLN derived from CCR7-/- animal; possibly due to impaired migration of kDC; the DCs present in rLN showed no significant difference in the amount of captured DQ-OVA (Fig. 22 B). These findings verify that rapid antigen transport must have occurred cell-independently.

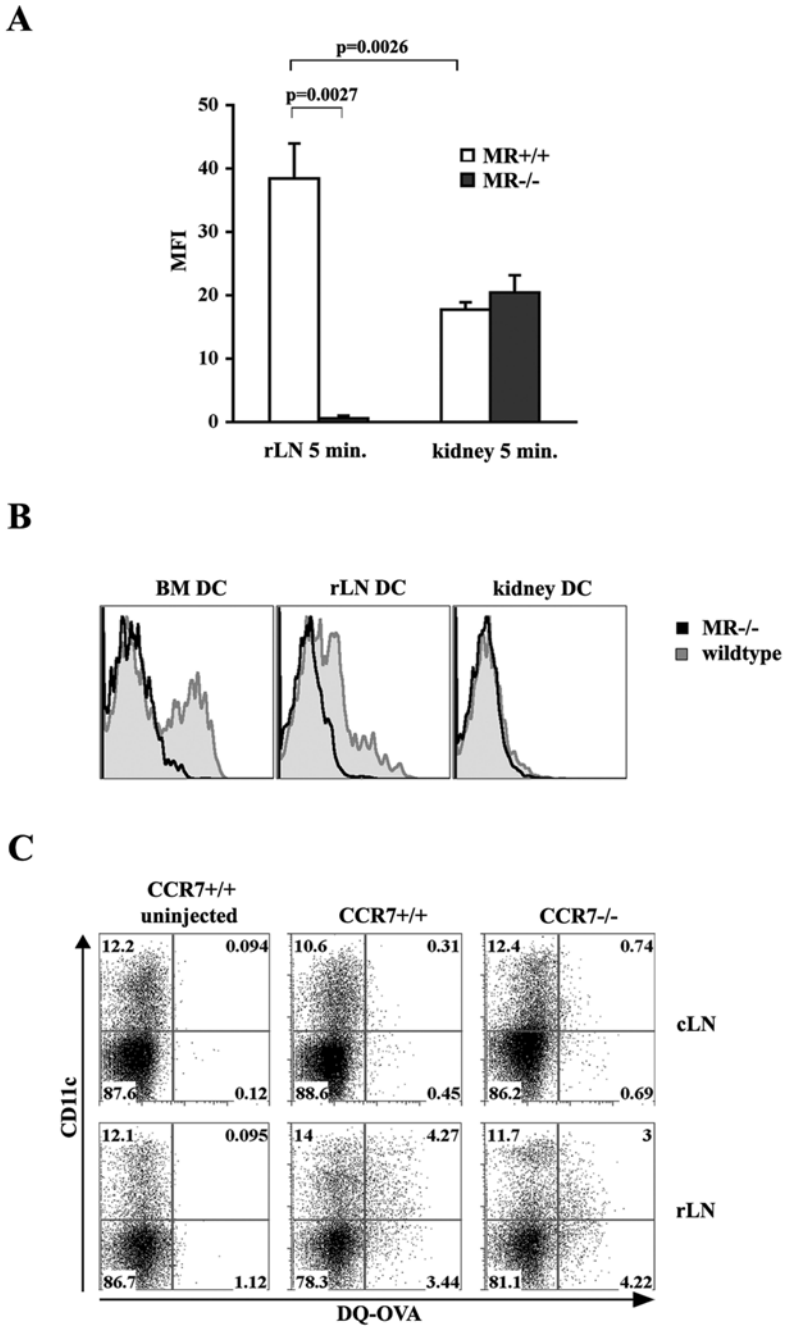

Figure 22. Antigen uptake in rLNs depends on the MR, but not on CCR7

A $MR^{-/-}$ mice or wildtype mice were injected *i.v* with 6 µg/g body weight aOVA. After 5 min, $CD11c^{+}$ cells in kidney and rLN were analyzed for aOVA MFI (n=3). Background fluorescence intensity detected in DC from non-injected mice was substracted from the MFI values.

B From $MR^{-/-}$ and wildtype mice DC were isolated from kidney, rLN and BM and stained intracellularly for MR.

C $CCR7^{-/-}$ or wildtype mice were injected *i.v* with 8 µg/g body weight OVA-DQ. After 1 h, total rLN were analyzed for CD11c expression and antigen uptake. Results are representative for 2 individual experiments.

7.2.3 Biased cross-presentation in the rLN towards filtrated antigen

To investigate whether filtrated antigen was presented to T cells, mice were injected with OVA and CFSE-labeled transgenic OVA-specific $CD8^+$ T cells (OT-I cells), which have been widely used for detecting cross-presentation of OVA (25). Indeed, OT-I cells proliferated more vigorously in rLNs than in cLNs in a dose dependent manner (Fig. 23 A, B). After injection of 10 μg OVA per g body-weight, the division index was 3 times higher in rLN than in cLN (Fig. 23 A, B), consequent with the difference found in the uptake of aOVA in these LNs (Fig. 20 A, B). Also higher aOVA doses were presented more efficiently in rLNs, whereas doses lower than 5 μg/g body weight did not cause significant T cell activation in any LN (Fig. 23 A, B). OT-I cell proliferation in the spleen was always higher than in rLNs (Fig. 23 A, B), in harmony with the efficient OVA uptake by splenic DC (Fig. 15).

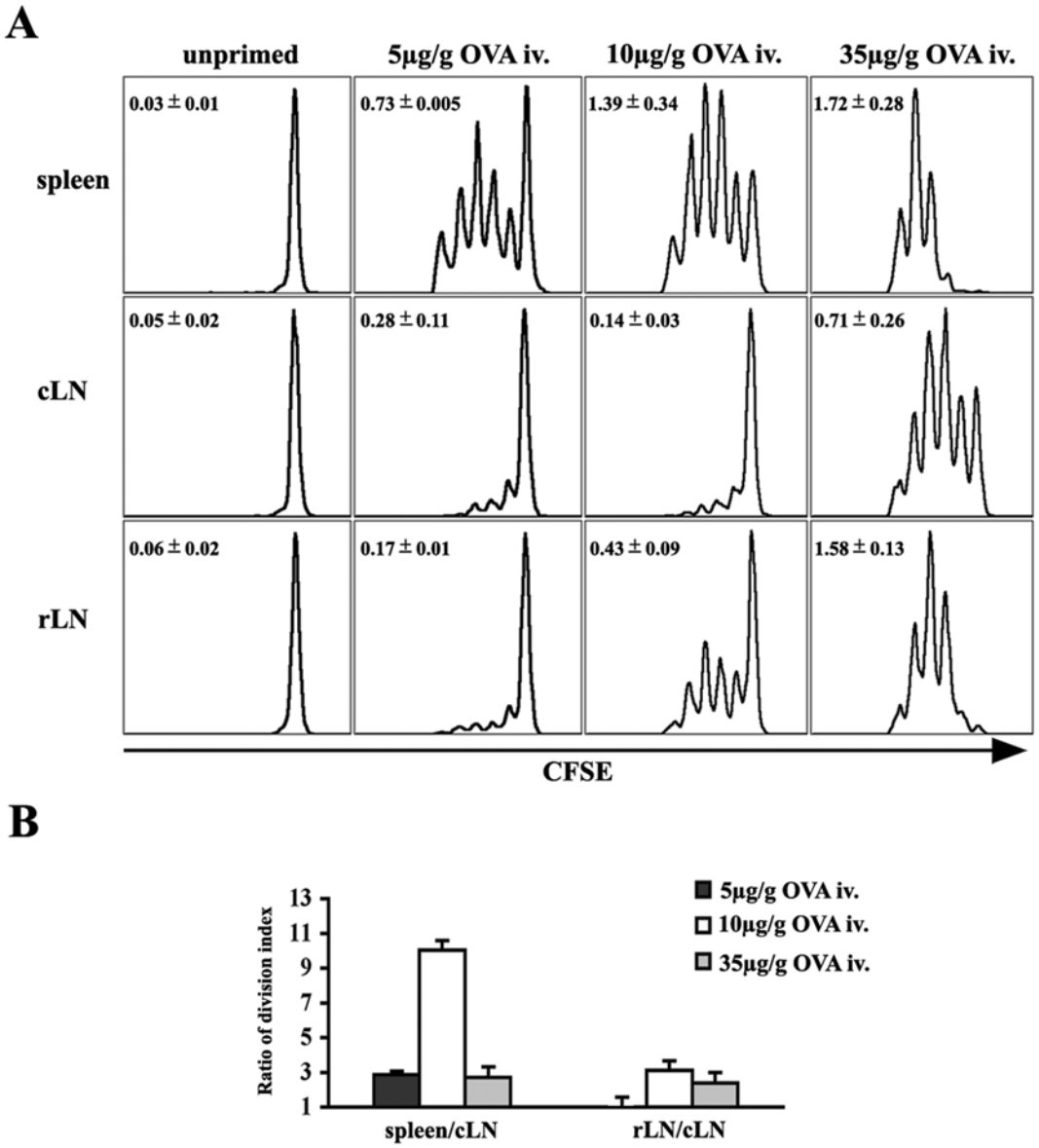

Figure 23. Biased cross-presentation in the rLN towards filtrated antigen

A $2x10^6$ CFSE-labeled OT I cells were injected into C57Bl/6 mice. 18 h later, different doses of OVA were *i.v.* injected. 48 hrs after OVA injection, the proliferation of OT I cells was analyzed in spleen, cutaneous and rLN. Histogram shows the proliferation profiles of $CFSE^+CD8^+$ cells. The numbers indicate the division index ± SD (n=3 mice)

B Statistical analysis of Fig. 23 A. The ratio between the division indices in the spleen or in rLNs divided by that of the cLN was given as a quantitative parameter for the superiority of spleen and rLNs in proliferation of soluble antigen at different doses.

7.2.4 T cell activation by filtrated molecules leads to tolerance

7.2.4.1 T cells activated by filtrated antigen in the rLN show a tolerized phenotype

To investigate the fate of T cells activated by filtrated antigen, activation markers on OT-I cells proliferating in response to such antigen were examined. Expression of CD44 was increased, and CD62L was decreased when compared to naive OT-I cells (Fig. 24). However, these activation signs were much smaller than those displayed by OT-I cells proliferating in response to OVA in complete Freund's adjuvant (OVA/CFA), which served as control for immunogenic priming (Fig. 24). In particular, these cells upregulated the high affinity IL-2 receptor alpha chain, CD25, whereas those in the rLN showed levels hardly above those of naive OT-I cells (Fig. 24).

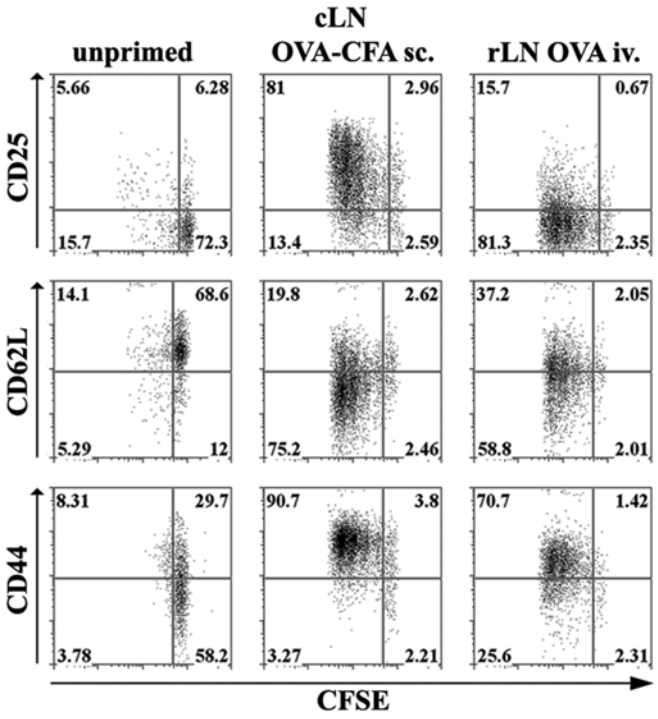

Figure 24. T cells activated by filtrated antigen in rLN show a tolerized phenotype
$2x10^6$ CFSE-labeled OT I cells were injected into C57Bl/6 mice. After 18 h, either 10µg/g body weight of OVA was injected *i.v.* or OVA-CFA *s.c.* 48 h later, expression of CD44, CD25, and CD62L on OT-I cells proliferating in response to these two antigen forms or in unprimed control mice were determined.

Consistent with this surface phenotype, OT-I cells; activated by filtrated antigen; after restimulation produced little IFNγ, and hardly any IL-2, whereas OT-I cells responding to OVA/CFA produced both cytokines in high amounts (Fig. 25 A, B). In order to further characterize the tolerogenic phenotype it was determined whether the OT-I cells could develop OVA-specific cytotoxic effector function. Priming with soluble OVA cytotoxicity was evident neither in rLNs, nor in any other location (Fig. 26). In contrast, cytotoxicity was detected in the draining cLN of mice *s.c.* injected with OVA/CFA, and to a lower extent in other LN and in the spleen (Fig. 26), presumably due to recirculation of activated OT-I cells.

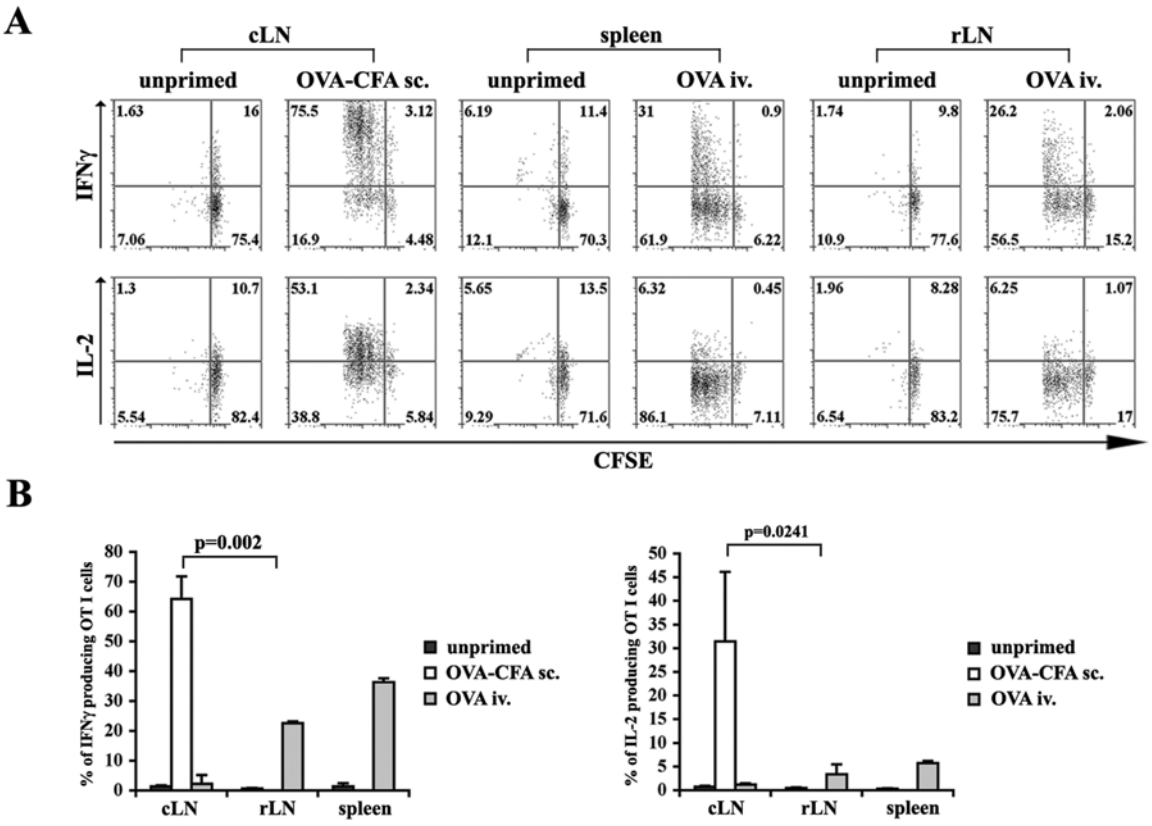

Figure 25. T cells activated by filtrated antigen in rLN showed impaired production of cytokines

A Intracellular IL-2 and IFNγ staining of CFSE-labeled OT-I cells proliferating in response to filtrated OVA or to OVA/CFA as described in Fig 24.

B Quantitative analysis of Fig. 25 A, showing the proportions of cytokine-producing OT-I cells in different organs (3-4 mice per group)

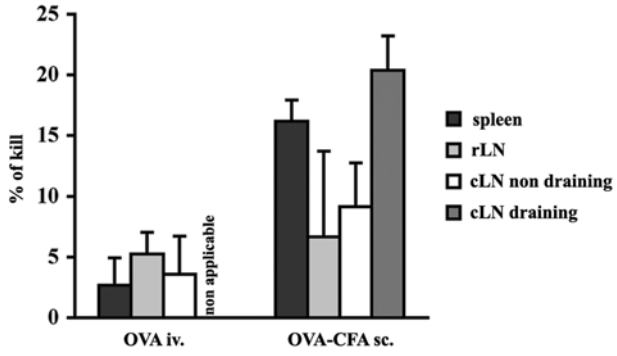

Figure 26. T cells activated by filtrated antigen show no cytotoxicity
Mice were injected with $1x10^4$ naive OT I cells and after 18 h, with either 10 µg/g body weight of soluble OVA *i.v.* or of OVA-CFA *s.c.* 5 d later, target cells were injected and 4 h later specific *in vivo* cytotoxicity was determined in different organs (n=3 mice)

7.2.4.2 T cells tolerized in the rLN are unable to participate in homeostatic proliferation presumably due to their deletion

Finally, the life span of T cells activated by filtrated antigen was determined. To this end, OT-I cells activated in the rLN of OVA-injected mice were transferred into $RAG^{-/-}$ secondary recipient mice, in order to subsequently determine their survival (experimental plan in Fig. 27 A). However, transfer of OT-I cells separated from the other cells in rLNs was impossible, because the very small cell numbers in the tiny rLN (usually in case of OVA primed animals 0.8-1.4 % of total living cells were OT-I cells) precluded all isolation or sorting attempts. To overcome this technical problem, we transferred total rLN cells pooled from several mice, and subsequently examined the proportion of OT-I cells within all transferred $CD8^+$ T cells. As control for immunogenic priming, draining cLN cells from OT-I cell-injected mice primed *s.c.* with OVA/CFA were transferred. A further control for naive OT-I cells was necessary, because rLNs contained not only OT-I cells activated by filtrated antigen, but also non-activated OT-I cells (Fig. 23, 24, 25) that presumably had not yet encountered OVA-presenting DC after entering the node. To this end, rLN cells were transferred from mice injected with OT-I cells, but not with OVA.

OT-I cells were more abundant in mice primed with soluble OVA or OVA/CFA than in unprimed animals (Fig. 23), consistent with their proliferation in these animals after priming in particular to OVA/CFA (Fig. 23). Two weeks after transfer into secondary recipients, the frequency of OT-I cells primed by filtrated antigen was reduced to the level of naive OT-I

cells (Fig. 27 B). In contrast, the frequency of OT-I cells activated by OVA/CFA had not declined (Fig. 27 B), indicating that immunogenically primed OT-I cells had been programmed to survive, as opposed to those primed by filtrated antigen. The loss of antigen specific OT-I cells was antigen specific, because the frequency of $V\alpha2^{+}$ $V\beta5^{-}$ $CD8^{+}$ cells, which were examined as examples for non-OVA-specific $CD8^{+}$ T cells, was not significantly altered in any experimental group (unprimed 6.6±2.8, filtrated antigen 3.5±1.4, OVA/CFA 3.3± 0.3 % of total $CD8^{+}$ T cells, n=3, $p>0.17$ for all comparisons). Taken together, OT-I cells primed in response to filtrated soluble antigen not only showed a tolerized phenotype, but also lacked cytotoxic activity and showed a reduced life-span, indicating induction of cross-tolerance.

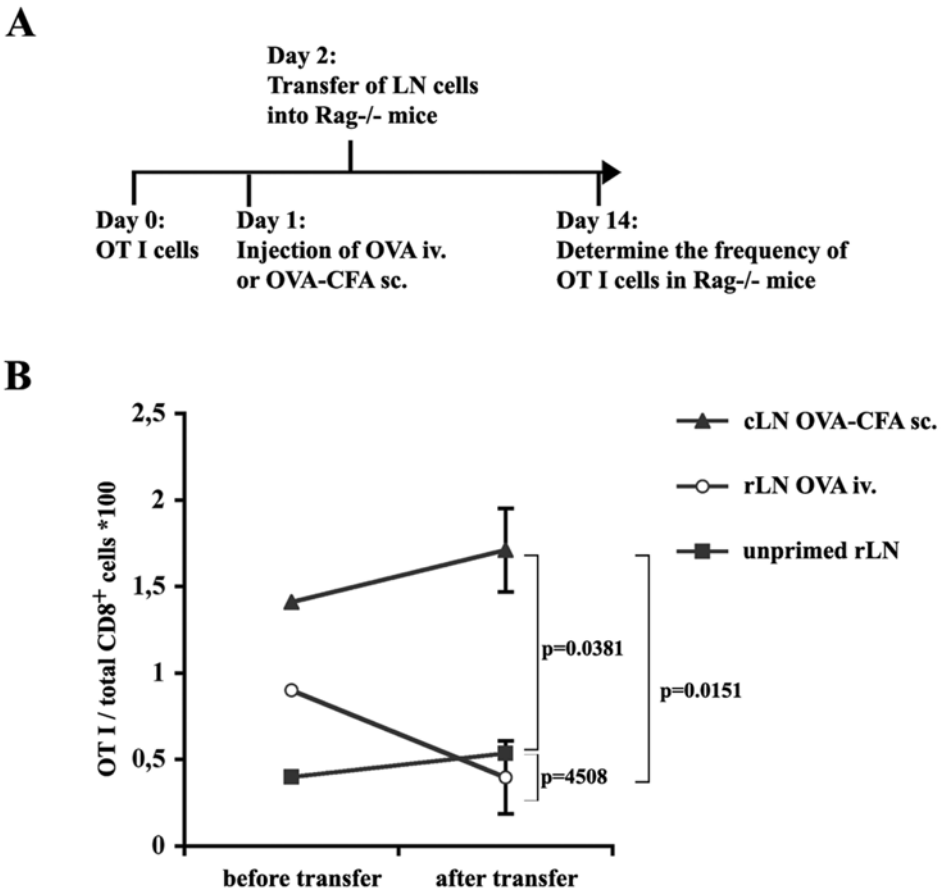

Figure 27. T cells activated by filtrated antigen in rLNs are deleted

A Experimental plan: $2x10^6$ CFSE-labeled OT I cells were injected into C57Bl/6 mice. After 18 hrs, mice were injected with either 10 µg/g of OVA *i.v.* or OVA-CFA *s.c.* After 48 h, single cell suspensions from the rLNs or the cLNs were transferred into $RAG^{-/-}$ secondary recipient mice. rLN cells were pooled from 6-8 first recipient mice. 12 d later, spleen and LN cells from $RAG^{-/-}$ mice were analyzed for the frequency of OT-I cells.

B Proportions of $V\alpha2^+$ $V\beta5^+$ OT-I cells in all $CD8^+$ cells before and after transfer into $RAG^{-/-}$ mice. (2 experiments using 2-3 mice per group).

7.2.5 Steady state migration of kDCs is not required for inducing T cell activation and tolerance induction towards filtrated antigen in rLN

In spite of the fast cell-independent enrichment of filtrated antigen in the rLN, kidney DC also demonstrated remarkable uptake capacity towards molecules filtrated in the kidney. Therefore the question should be raised whether the CD8 T cell response; induced in rLN; was mediated by LN-resident DC or by kidney DC involved in steady-state migration. The latter possibility would involve that filtrated antigens are taken up in the kidney and transported to draining LN by kDCs. To distinguish between these two possibilities, proliferation of OT-I cells was examined in mice unilaterally nephrectomized after injection of OVA. Nephrectomy was carried out 90 min after antigen injection, because this time had been reported to suffice for clearing the murine plasma of a single bolus injection of filterable molecules (186). Importantly, during this time period no significant amount of antigen accumulation was found in the cLN (Fig. 28), indicating that antigen enriched in rLN predominantly derived from the kidney and not from the circulation. Indeed, OT-I cell proliferation was similar in the ipsilateral and contralateral rLN of nephrectomized mice, and in sham-operated animals (Fig. 29 A, B), indicating that rapidly transported antigen was sufficient to drive T cell proliferation. Hypothetical DC-mediated steady-state antigen transport at later time points was not required.

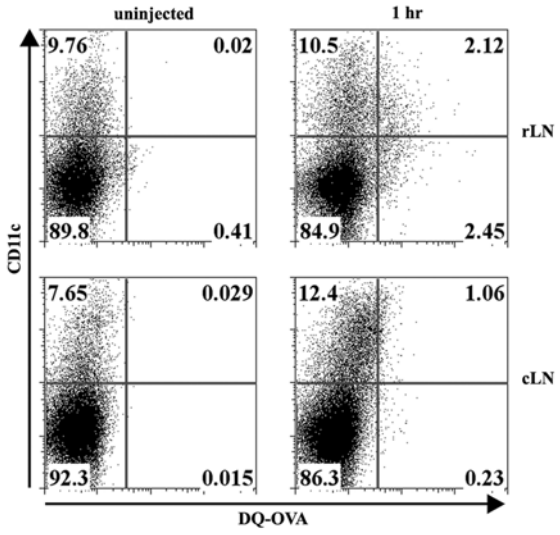

Figure 28. Presence of DQ-OVA 1 hr after injection into wildtype mice
Wildtype mice were injected *i. v.* with 8µg/g body weight DQ-OVA. 1 hr after injection cLN and rLN were analyzed for CD11c expression and OVA uptake. Cells shown are PI negative.

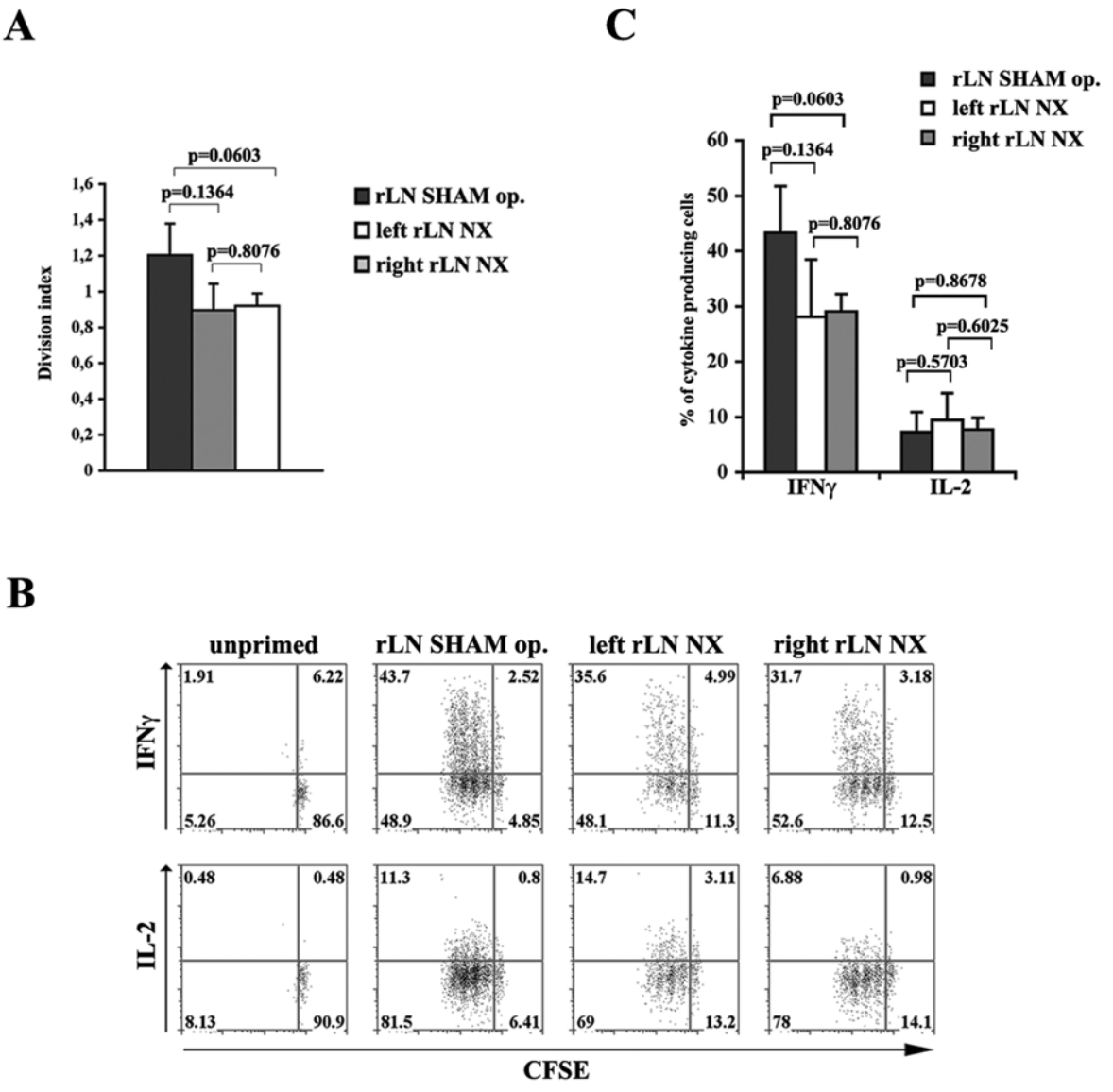

Figure 29. Effect of steady state DC migration on the T cell response against filtrated antigen

A Left nephrectomy (NX) was performed 90 min after injection of OVA. Division indices of proliferating OT-I cells were determined in the left and right rLN after left nephrectomy (NX), and in the left rLN of sham-operated animals. (n=6 mice per group, rLNs from 2 mice were pooled for analysis).

B Same experimental setup as in Fig. 29 A, shown are the intracellular IL-2 and IFNγ content of OT-I cells proliferating in the left and right rLN and in the left rLN of sham-operated animals.

C Quantitative analysis of Fig 29 B, showing the proportions of cytokine-producing OT-I cells (6 mice per group, rLNs from 2 mice were pooled for analysis).

Furthermore, OT-I cells produced low cytokine amounts also when mice were unilaterally nephrectomized 90 min after OVA injection (Fig. 29 B, C). Thus, rapidly transported antigen presented by LN resident DC was sufficient for imprinting OT-I cells for defective cytokine production, demonstrating once more that hypothetical DC-mediated antigen transport at later time points was not required.

8. Discussion

8.1 Part 1: The mannose receptor mediates uptake of soluble, but not of cell-associated antigen for cross-presentation

The MR is best known for its role as a scavenger receptor active in macrophages. A role in antigen presentation has been proposed by others based on its expression by human DC *in vitro,* and on its role in the uptake of mannosylated structures such as dextranes or horseradish peroxydase in a mannan-blockable fashion (95, 121, 187). Although, mannan and mannosylated proteins bind with high affinity to MR, they can also bind to other C-type lectin receptors such as DC-SIGN (127) that could also contribute to the antigen presentation observed in above studies. To circumwent the specificity concerns MR deficient mice were used to investigate the role of the MR in antigen presentation.

The present study showed that the MR predominantly mediated the uptake of soluble OVA by DC *in vivo*. Concomitantly, in MR deficient mice the activation of naive CD8 T cells by soluble OVA was diminished. This was most likely due to reduced cross-presentation of OVA, because the absence of the MR did not affect the expression of costimulatory molecules on DC. Reduced recirculation of activated OT I cells was also ruled out as alternative explanation by showing that OT I cell activation was unaltered in $MR^{-/-}$ mice responding to cell-associated antigen. Also differences in the DC subtype composition of MR-deficient mice were unchanged, in particular the content of $CD8\alpha^+$ DC, which have been reported to mediate *in vivo* cross-presentation of soluble and cell associated OVA (37, 39). The MR was abundant in freshly isolated DC of the spleen, bone marrow and LN, despite one previous early report showing lack of MR expression (131). As opposed to the immunohistochemical detection of MR in that study (131), here a more sensitive method was used: intracellular staining and analysis by flow cytometry. This allowed the detection of low expression levels of the MR in DCs. Therefore the discrepancy to the previous study is most likely due to technical differences and limitations in detection sensitivity. Importantly, the MR was exclusively found in $CD8\alpha^+$ DC of the spleen, suggesting it may be used as a further marker for cross-presenting

APC *in vivo* besides CD8α (37) and DEC-205 (185). Only $CD8\alpha^+$ DC were capable of cross-presntation of soluble antigen, although both $CD8\alpha^+$ and $CD8\alpha^-$ DC were able to aquire antigen (39). These findings suggested that the cross-presentation ability is differentially regulated within the subsets. One hypothesis of the present study is that the receptor that mediates uptake of a given antigen may determine its intracellular routing and its presentation to T cells. This notion was verified by showing that the MR could target the antigen to the cross-presentation pathway. This mechanism may be complimentary to the recent finding that $CD8\alpha^+$ DC have higher expression of MHC I processing machinery compared to $CD8\alpha^-$ ones (185).

In contrast to soluble OVA, uptake of cell-associated OVA and the resulting activation of OT-I cells were not impaired by the absence of the MR. The cell-associated antigen used in the present study was actively taken up by the bm1 cells, at least in part (Fig. 9). These cells before injection were UV irradiated in order to induce apoptosis. The efficient elimination of dying or death cells *in vivo* is crucial for tissue homeostasis. The surface molecules present on apoptotic cells and their receptors expressed by phagocytes form the phagocytic synapse (91). Many receptors have been considered to be involved in the formation of such synapse such as complement-, scavenger-, PS receptor and different integrins (3, 91). The role of some of these receptors was investigated in gene deficient animals such as CD36 knockout mice, but no essential role in the clearance of apoptotic cells has been observed yet (90). This has been explained by functional redundancy between these receptors. The present study excluded the MR as one such receptor. Thus, the mechanisms mediating antigen uptake for classical cross-priming as described by Bevan (36) remain to be resolved.

Taken together, the present study provided formal *in vivo* evidence that a particular uptake receptor, here the MR, can specifically introduce antigen (soluble OVA) into the cross-presentation pathway.

These findings suggest that *in vivo* targeting of the MR may be useful for introducing extracellular antigen into the MHC-class I-restricted antigen presentation pathway. Targeting of a related lectin, DEC205, has been shown to result in tolerogenic CD4 and CD8 T cell activation (100). It will be interesting to investigate whether antigen uptake by the MR would result in immunogenic or tolerogenic CTL priming, or in both, depending on the absence or presence of inflammatory stimuli. Finally, paralell work on BM-DC *in vitro* carried out by Dr.

Sven Burgdorf (182) in Prof. Kurts`s laboratory has shown that in these cells uptake and cross-presentation of soluble OVA was completely abrogated in MR deficient DCs. Using this *in vitro* system may allow to understand which intracellular mechanisms are involved and how MR target the cross-presentation pathway.

8.1.1 Summarized features of the cross-presenting APC

The present study provides evidences that an antigen uptake receptor, the MR, targets antigen specifically into the cross-presentation pathway. These results extend the characteristic features of the cross-presenting APC in the spleen. These properties are summarized in Table 2.

Cross-presentation	+++	-	(36)
CD8α	+	-	16, 37, 39)
DEC-205	+	-	(13, 100, 185)
DCIR2	-	+	(185)
MR	+	-	(182)

Table 2. Summary of the surface markers, which are characteristic of the cross-presenting DCs in spleen

8.2 Part 2: Lymph node-resident dendritic cells induce T cell cross-tolerance against tissue-derived antigen

Systemic injection of soluble antigen devoid of inflammatory stimuli has been widely used to study mechanisms of T and B cell tolerance (178, 180, 181). This system is thought to mimic presentation of innocuous self-serum proteins and orally administered antigen, against which immunity is not required, or may even be harmful. It is generally assumed that such tolerance is induced in the spleen, which presents soluble antigens to T cells particularly well (75, 143, 174, 188). However, splenectomy usually does not result in auto-immunity against otherwise ignored soluble antigens, suggesting that further sites may exist where tolerance can be induced. One possible such site may be the kidney-rLN system because of the physiologic filtration of circulating molecules below albumin size in kidney glomeruli. Liquid reabsorption from the glomerular ultrafiltrate causes enrichment of such molecules in the kidney tubular system (27). Unlike metabolic waste products, filterable proteins are not excreted with the urine, but instead are reabsorbed by tubular epithelial cells. Dong *et al.* recently reported that filterable dextran molecules were taken up also by kDC (23). In the present study, simultaneous injection of small and large fluorescent molecules allowed demonstrating that their uptake from the tubular filtrate was far more efficient than that from the circulation. Furthermore, it revealed selective enrichment of filterable protein antigen in kDC and rLN DC. It has been reported that tubular cells of the kidney regularly sense filtrated proteins, such as albumin and growth factors and that they can respond with secretion of chemokines that might affect renal diseases (35). What consequences filtrated proteins exert on DCs and what role they play in renal diseases remained to be elucidated. This open question was addressed in the present study by showing that their enrichment in the rLN resulted in efficient activation of naive $CD8^+$ T cells at concentrations too low for priming in any other LN. Also the spleen enriched soluble antigen, and even more efficiently so than rLNs. This observation was consistent with the previous identification of a splenic conduit system that efficiently targeted soluble small molecules to the T cell/DC areas (143). Also LNs (141) and the thymus (142) contain conduit systems. Such a system may also have conveyed filtrated antigen arriving with the lymph flow towards resident DC within rLNs. In addition to such anatomical structures,

the present study reveals that concentration of filterable antigen in the kidney acts as a functional mechanism for improving presentation of soluble antigen.

Presentation of filtrated antigen induced $CD8^+$ T cell cross-tolerance, as evidenced by low activation markers, such as CD25. The lack of upregulation of this receptor is often considered a phenotypic feature of tolerized T cells for example after activation by autoantigen (174). Tolerance induction was further supported by the lack of significant cytokine production, for example of IFN-γ and IL-2, and by the absence of cytotoxic activity. Finally, the life span of the activated OT-I cells was determined. Demonstrating this latter point was technically demanding, because rLNs contained not only activated, but also naive OT-I cells that had not yet been tolerized. The extremely low cell numbers in this node precluded separation of naïve and activated OT-I cells for determining their individual life spans. Nevertheless, transfer of unseparated OT-I cells to Rag-/- recipients allowed demonstrating their decline to the level observed after transfer of naïve OT-I cells. Suggesting that OT-I cells primed in renal LN with filtrated soluble antigen were unable to participate in homeostatic proliferation after transfer into immunodeficient recipients. This suggested that activated OT-I cells had been deleted, while the naïve ones survived. Induction of anergy as an alternative explanation to deletion is unlikely, because others showed that transfer into $RAG^{-/-}$ mice rescued anergic T cells, and enabled vigorous homeostatic proliferation (189). Thus, the observed decline of OT-I cells activated by filtrated antigen most likely indicates their deletion as most likely explanation. Thus, the rLN is a unique site where deletional T cell-tolerance against filterable antigen is induced.

The selectivity of enrichment for filterable molecules in kDC and in rLN DC allowed studying how soluble antigen was transported from the kidney to the rLN. Strikingly, several lines of independent evidence indicated that this transport did not occur via steady-state DC migration. First, enrichment of filtrated antigen was detectable already 90 sec after antigen injection. Moreover, 5 min after injection, $40\text{-}50x10^3$ of DC carrying filtrated antigen were present in the left kidney, whereas the left rLN contained $2\text{-}3x10^3$ DC (Fig. 21 B). If all these rLN DC had arrived from the kidney within this short time-span, then more than $500x10^3$ DC would migrate per day. In this case, kDC would turn over more than 10 times and rLN DC more than 200 times per day, which is clearly incompatible with reports by others showing DC half-lifes of 1.5-2.9 days in the spleen (190) and >2 weeks in the kidney (23). These estimations of DC

turnover, together with the remarkably rapid kinetics of antigen relocation are not compatible with low-level steady-state DC migration over longer time spans.

The observed phenotypical differences between DC carrying filtrated antigen in the kidney and in the rLN provide the second argument against DC migration. Downregulation of CD11c and upregulation of $CD8\alpha^+$ in such short time appears unlikely. Besides, $CD8\alpha^+$ is considered a lineage marker that is not expressed *de novo* on conventional DC (16, 18, 20), such as those residing in the kidney (21). The absence of $CD8\alpha^+$ DC from the kidney demonstrated that the few $CD8\alpha^+$ DC carrying filtrated antigen in the rLN couldn't have immigrated from this organ. Third, most rLN DC rapidly took up large antigen amounts via the MR. Also these DC could not be kidney-derived, because this organ contained no such DC either. Fourth, the rapid accumulation of filtrated OVA in rLN DC was independent of CCR7, which is required for DC trafficking (8, 153, 154) to draining LNs and which is expressed by kDC (26). These four findings together can be explained only by cell-independent antigen transport to rLNs and subsequent uptake by LN-resident DC, which then induced cross-tolerance.

Cell-independent antigen transport from peripheral tissues to draining LNs differs from previously described mechanisms of peripheral T cell tolerance via steady-state migratory DC (8, 17, 45). Previous studies relied on various approaches to label migratory DC, for example local labeling by FITC-painting, injection of fluorochromes or latex beads, or of labeled DC (140). It is difficult in any of these approaches to exclude that the labeling process itself leads to DC maturation and triggers migration. The approach of *i.v.* injection of tracers in this work *bona fide* allowed physiologic labeling of DC and reproducibly did not result in any detectable maturation. Under these conditions, tolerance was induced without DC migration. While these finings do not exclude DC-mediated antigen transport to rLNs at later times points, but nephrectomy experiments in the present study demonstrated (Fig. 29) that such hypothetical steady state migration was not required for T cell activation and tolerization in this system.

Recently, stromal LN cells that expressed the *Aire* gene as well as peripheral self-antigens have been shown to tolerize autoreactive $CD8^+$ T cells (179). DC-independent relocation of antigen to LN-resident DC indicates a further mechanism of tolerance induction in secondary lymphatics. Others have previously shown antigen transfer to LN-resident DC from migratory DC (17). This antigen transport mechanism has been documented in models of herpes virus infection in the skin and in the lung resulted in immunogenic T cell activation (57, 151). Apart

from the organ studied, this system differs from above findings by the presence of an infectious agent that could mature DC and induce their migration. In contrast, when innocuous antigen was injected into the skin, it reached the LN through lymph vessels before skin-derived DC had immigrated (148-150). Also the kidney is connected with its draining LN by lymph vessels, as demonstrated by cannulating experiments in sheep (191). Cannulation of the tiny lymph vessels of mice, however, has not been achieved yet. At least in rats, renal lymphatic vessels have been microscopically observed, albeit only after ureteral obstruction, when lymph vessels were blocked and enlarged by lymphatic fluid (192). In human renal biopsies, lymphatic vessels were large enough to be microscopically detectable in healthy organs (193). Such vessels may anatomically explain how filtrated antigen was relocated in a cell-independent manner from the kidney to its LN, even though above study does not provide direct evidence for lymph vessel mediated antigen transport.

Nevertheless, these results demonstrate that DC-independent relocation of innocuous circulating antigen from the kidney to the rLN and local uptake by resident DC can result in T cell tolerance. This mechanism may contribute to prevent unwanted immunity against self-serum proteins or innocuous orally administered antigen.

8.2.1 Model of handling of filtrated molecules in the kidney-renal LN system

Taken together the above results support the following model (Fig. 30): molecules below of albumin size are concentrated in the kidney due to their filtration in the glomeruli. These filtrated molecules are transported rapidly via cell-independent mechanism to the draining renal LN. These molecules are endocytosed by LN resident DCs. Presentation of filtrated and accumulated antigen in renal LN results in a biased cross-presentation, which in turn causes tolerogenic CD8 T cell activation. Therefore, the kidney–renal LN system could participate in elimination of autoreactive CD8 T cells against small, filterable self-proteins such as serum proteins or food-antigens.

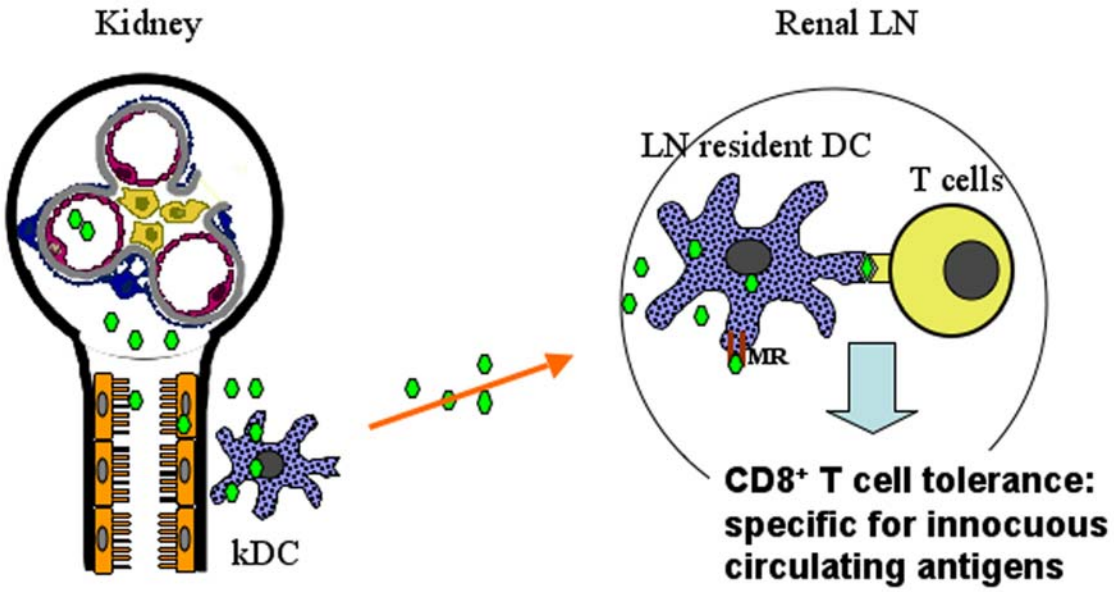

Figure 30. Model of handling filtrated molecules by the kidney-renal LN system

9. References

1. Charles A. Janeway, P. T., Mark Walport, Mark J. Shlomchik. 2001. *Immunobiology*.
2. Wilson, N. S., D. El-Sukkari, G. T. Belz, C. M. Smith, R. J. Steptoe, W. R. Heath, K. Shortman, and J. A. Villadangos. 2003. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood 102:2187*.
3. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol 20:621*.
4. Trombetta, E. S., and I. Mellman. 2005. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol 23:975*.
5. Kapsenberg, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol 3:984*.
6. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell 106:255*.
7. Vermaelen, K. Y., I. Carro-Muino, B. N. Lambrecht, and R. A. Pauwels. 2001. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med 193:51*.
8. Hintzen, G., L. Ohl, M. L. del Rio, J. I. Rodriguez-Barbosa, O. Pabst, J. R. Kocks, J. Krege, S. Hardtke, and R. Forster. 2006. Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. *J Immunol 177:7346*.
9. Huang, F. P., N. Platt, M. Wykes, J. R. Major, T. J. Powell, C. D. Jenkins, and G. G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med 191:435*.
10. Chung, Y., J. H. Chang, M. N. Kweon, P. D. Rennert, and C. Y. Kang. 2005. CD8alpha-11b+ dendritic cells but not CD8alpha+ dendritic cells mediate cross-tolerance toward intestinal antigens. *Blood 106:201*.
11. Kurts, C., H. Kosaka, F. R. Carbone, J. F. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med 186:239*.
12. Hernandez, J., S. Aung, W. L. Redmond, and L. A. Sherman. 2001. Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J Exp Med 194:707*.
13. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol 21:685*.
14. Steinman, R. M. 2003. Some interfaces of dendritic cell biology. *Apmis 111:675*.
15. Munz, C., R. M. Steinman, and S. Fujii. 2005. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med 202:203*.
16. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol 2:151*.
17. Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol 7:19*.

18. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol 164:2978*.
19. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev 199:9*.
20. Naik, S., D. Vremec, L. Wu, M. O'Keeffe, and K. Shortman. 2003. CD8alpha+ mouse spleen dendritic cells do not originate from the CD8alpha- dendritic cell subset. *Blood 102:601*.
21. Kruger, T., D. Benke, F. Eitner, A. Lang, M. Wirtz, E. E. Hamilton-Williams, D. Engel, B. Giese, G. Muller-Newen, J. Floege, and C. Kurts. 2004. Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. *J Am Soc Nephrol 15:613*.
22. Soos, T. J., T. N. Sims, L. Barisoni, K. Lin, D. R. Littman, M. L. Dustin, and P. J. Nelson. 2006. CX3CR1+ interstitial dendritic cells form a contiguous network throughout the entire kidney. *Kidney Int 70:591*.
23. Dong, X., S. Swaminathan, L. A. Bachman, A. J. Croatt, K. A. Nath, and M. D. Griffin. 2005. Antigen presentation by dendritic cells in renal lymph nodes is linked to systemic and local injury to the kidney. *Kidney Int 68:1096*.
24. Coates, P. T., F. J. Duncan, B. L. Colvin, Z. Wang, A. F. Zahorchak, W. J. Shufesky, A. E. Morelli, and A. W. Thomson. 2004. In vivo-mobilized kidney dendritic cells are functionally immature, subvert alloreactive T-cell responses, and prolong organ allograft survival. *Transplantation 77:1080*.
25. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med 184:923*.
26. Coates, P. T., B. L. Colvin, A. Ranganathan, F. J. Duncan, Y. Y. Lan, W. J. Shufesky, A. F. Zahorchak, A. E. Morelli, and A. W. Thomson. 2004. CCR and CC chemokine expression in relation to Flt3 ligand-induced renal dendritic cell mobilization. *Kidney Int 66:1907*.
27. Tryggvason, K., and J. Wartiovaara. 2001. Molecular basis of glomerular permselectivity. *Curr Opin Nephrol Hypertens 10:543*.
28. Abbate, M., and G. Remuzzi. 2001. Novel mechanism(s) implicated in tubular albumin reabsorption and handling. *Am J Kidney Dis 38:196*.
29. Matthew N. Levy, B. M. K., Bruce A. Stanton. 2005. *Berne &Levy Principles of Physiology, 4th edition*.
30. Eppel, G. A., T. M. Osicka, L. M. Pratt, P. Jablonski, B. Howden, E. F. Glasgow, and W. D. Comper. 2001. The return of glomerular filtered albumin to the rat renal vein--the albumin retrieval pathway. *Ren Fail 23:347*.
31. Gudehithlu, K. P., A. A. Pegoraro, G. Dunea, J. A. Arruda, and A. K. Singh. 2004. Degradation of albumin by the renal proximal tubule cells and the subsequent fate of its fragments. *Kidney Int 65:2113*.
32. Brunskill, N. J. 2004. Albumin signals the coming of age of proteinuric nephropathy. *J Am Soc Nephrol 15:504*.

33. Diwakar, R., A. D. Pearson, P. Colville-Nash, N. J. Brunskill, and M. E. Dockrell. 2007. The Role Played by Endocytosis in Albumin Induced Secretion of TGF{beta}-1 by Proximal Tubular Epithelial Cells. *Am J Physiol Renal Physiol*.
34. Tang, S., J. C. Leung, K. Abe, K. W. Chan, L. Y. Chan, T. M. Chan, and K. N. Lai. 2003. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *J Clin Invest 111:515*.
35. Wang, S. N., J. LaPage, and R. Hirschberg. 2000. Role of glomerular ultrafiltration of growth factors in progressive interstitial fibrosis in diabetic nephropathy. *Kidney Int 57:1002*.
36. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med. 143:1283*.
37. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med 192:1685*.
38. den Haan, J. M., and M. J. Bevan. 2002. Constitutive versus Activation-dependent Cross-Presentation of Immune Complexes by CD8(+) and CD8(-) Dendritic Cells In Vivo. *J Exp Med 196:817*.
39. Pooley, J. L., W. R. Heath, and K. Shortman. 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*.
40. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R. M. Steinman, and K. Inaba. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med 195:1289*.
41. Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. *J Immunol 172:1996*.
42. Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol 175:196*.
43. Pozzi, L. A., J. W. Maciaszek, and K. L. Rock. 2005. Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *J Immunol 175:2071*.
44. Kurts, C., M. Cannarile, I. Klebba, and T. Brocker. 2001. Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. *J Immunol 166:1439*.
45. Belz, G. T., G. M. Behrens, C. M. Smith, J. F. Miller, C. Jones, K. Lejon, C. G. Fathman, S. N. Mueller, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J Exp Med 196:1099*.
46. Norbury, C. C., D. Malide, J. S. Gibbs, J. R. Bennink, and J. W. Yewdell. 2002. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat Immunol 3:265*.
47. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman, and R. A. Lang. 2002. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity 17:211*.

48. Probst, H. C., K. Tschannen, B. Odermatt, R. Schwendener, R. M. Zinkernagel, and M. Van Den Broek. 2005. Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. *Clin Exp Immunol 141:398*.
49. Valdez, Y., W. Mah, M. M. Winslow, L. Xu, P. Ling, and S. E. Townsend. 2002. Major histocompatibility complex class II presentation of cell-associated antigen is mediated by CD8alpha+ dendritic cells in vivo. *J Exp Med 195:683*.
50. Bennett, S. R., F. R. Carbone, F. Karamalis, J. F. Miller, and W. R. Heath. 1997. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med 186:65*.
51. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478*.
52. Behrens, G. M., M. Li, G. M. Davey, J. Allison, R. A. Flavell, F. R. Carbone, and W. R. Heath. 2004. Helper requirements for generation of effector CTL to islet beta cell antigens. *J Immunol 172:5420*.
53. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature 421:852*.
54. Janssen, E. M., N. M. Droin, E. E. Lemmens, M. J. Pinkoski, S. J. Bensinger, B. D. Ehst, T. S. Griffith, D. R. Green, and S. P. Schoenberger. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature 434:88*.
55. Schulz, O., and C. Reis e Sousa. 2002. Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology 107:183*.
56. Schnorrer, P., G. M. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, G. T. Belz, F. R. Carbone, K. Shortman, W. R. Heath, and J. A. Villadangos. 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*.
57. Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone, and W. R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci U S A 101:8670*.
58. Kovacsovics-Bankowski, M., K. Clark, B. Benacerraf, and K. L. Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U S A 90:4942*.
59. Kovacsovics-Bankowski, M., and K. L. Rock. 1994. Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and II presentation and regulation by cytokines. *Eur J Immunol 24:2421*.
60. Debrick, J. E., P. A. Campbell, and U. D. Staerz. 1991. Macrophages as accessory cells for class I MHC-restricted immune responses. *J Immunol 147:2846*.
61. Ramirez, M. C., and L. J. Sigal. 2002. Macrophages and dendritic cells use the cytosolic pathway to rapidly cross-present antigen from live, vaccinia-infected cells. *J Immunol 169:6733*.

62. Chang, M. D., E. R. Stanley, H. Khalili, O. Chisholm, and J. W. Pollard. 1995. Osteopetrotic (op/op) mice deficient in macrophages have the ability to mount a normal T-cell-dependent immune response. *Cell Immunol 162:146*.
63. Delemarre, F. G., N. Kors, and N. van Rooijen. 1990. Elimination of spleen and of lymph node macrophages and its difference in the effect on the immune response to particulate antigens. *Immunobiology 182:70*.
64. Hon, H., A. Oran, T. Brocker, and J. Jacob. 2005. B lymphocytes participate in cross-presentation of antigen following gene gun vaccination. *J Immunol 174:5233*.
65. Tobian, A. A., C. V. Harding, and D. H. Canaday. 2005. Mycobacterium tuberculosis heat shock fusion protein enhances class I MHC cross-processing and -presentation by B lymphocytes. *J Immunol 174:5209*.
66. Brandes, M., K. Willimann, and B. Moser. 2005. Professional antigen-presentation function by human gammadelta T Cells. *Science 309:264*.
67. Potter, N. S., and C. V. Harding. 2001. Neutrophils process exogenous bacteria via an alternate class I MHC processing pathway for presentation of peptides to T lymphocytes. *J Immunol 167:2538*.
68. Tvinnereim, A. R., S. E. Hamilton, and J. T. Harty. 2004. Neutrophil involvement in cross-priming CD8+ T cell responses to bacterial antigens. *J Immunol 173:1994*.
69. Limmer, A., J. Ohl, C. Kurts, H. G. Ljunggren, Y. Reiss, M. Groettrup, F. Momburg, B. Arnold, and P. A. Knolle. 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*.
70. Limmer, A., J. Ohl, G. Wingender, M. Berg, F. Jungerkes, B. Schumak, D. Djandji, K. Scholz, A. Klevenz, S. Hegenbarth, F. Momburg, G. J. Hammerling, B. Arnold, and P. A. Knolle. 2005. Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur J Immunol 35:2970*.
71. Rock, K. L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev 207:166*.
72. Rock, K. L. 2006. Exiting the outside world for cross-presentation. *Immunity 25:523*.
73. Kurts, C., J. F. Miller, R. M. Subramaniam, F. R. Carbone, and W. R. Heath. 1998. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J Exp Med 188:409*.
74. Wolkers, M. C., N. Brouwenstijn, A. H. Bakker, M. Toebes, and T. N. Schumacher. 2004. Antigen bias in T cell cross-priming. *Science 304:1314*.
75. Li, M., G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone, and W. R. Heath. 2001. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J Immunol 166:6099*.
76. Norbury, C. C., S. Basta, K. B. Donohue, D. C. Tscharke, M. F. Princiotta, P. Berglund, J. Gibbs, J. R. Bennink, and J. W. Yewdell. 2004. CD8+ T cell cross-priming via transfer of proteasome substrates. *Science 304:1318*.
77. Shen, L., and K. L. Rock. 2004. Cellular protein is the source of cross-priming antigen in vivo. *Proc Natl Acad Sci U S A 101:3035*.
78. Serna, A., M. C. Ramirez, A. Soukhanova, and L. J. Sigal. 2003. Cutting edge: efficient MHC class I cross-presentation during early vaccinia infection requires the transfer of proteasomal intermediates between antigen donor and presenting cells. *J Immunol 171:5668*.

79. Basta, S., R. Stoessel, M. Basler, M. van den Broek, and M. Groettrup. 2005. Cross-presentation of the long-lived lymphocytic choriomeningitis virus nucleoprotein does not require neosynthesis and is enhanced via heat shock proteins. *J Immunol 175:796*.
80. Neijssen, J., C. Herberts, J. W. Drijfhout, E. Reits, L. Janssen, and J. Neefjes. 2005. Cross-presentation by intercellular peptide transfer through gap junctions. *Nature 434:83*.
81. Conner, S. D., and S. L. Schmid. 2003. Regulated portals of entry into the cell. *Nature 422:37*.
82. Norbury, C. C. 2006. Drinking a lot is good for dendritic cells. *Immunology 117:443*.
83. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol 2:361*.
84. Harshyne, L. A., M. I. Zimmer, S. C. Watkins, and S. M. Barratt-Boyes. 2003. A role for class A scavenger receptor in dendritic cell nibbling from live cells. *J Immunol 170:2302*.
85. Rubartelli, A., A. Poggi, and M. R. Zocchi. 1997. The selective engulfment of apoptotic bodies by dendritic cells is mediated by the alpha(v)beta3 integrin and requires intracellular and extracellular calcium. *Eur J Immunol 27:1893*.
86. Takizawa, F., S. Tsuji, and S. Nagasawa. 1996. Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. *FEBS Lett 397:269*.
87. Albert, M. L., S. F. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med 188:1359*.
88. Fadok, V. A., D. L. Bratton, D. M. Rose, A. Pearson, R. A. Ezekewitz, and P. M. Henson. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature 405:85*.
89. Schulz, O., D. J. Pennington, K. Hodivala-Dilke, M. Febbraio, and C. Reis e Sousa. 2002. CD36 or alphavbeta3 and alphavbeta5 integrins are not essential for MHC class I cross-presentation of cell-associated antigen by CD8 alpha+ murine dendritic cells. *J Immunol 168:6057*.
90. Belz, G. T., D. Vremec, M. Febbraio, L. Corcoran, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. CD36 is differentially expressed by CD8+ splenic dendritic cells but is not required for cross-presentation in vivo. *J Immunol 168:6066*.
91. Lauber, K., S. G. Blumenthal, M. Waibel, and S. Wesselborg. 2004. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell 14:277*.
92. Garrett, W. S., L. M. Chen, R. Kroschewski, M. Ebersold, S. Turley, S. Trombetta, J. E. Galan, and I. Mellman. 2000. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell 102:325*.
93. West, M. A., A. R. Prescott, E. L. Eskelinen, A. J. Ridley, and C. Watts. 2000. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol 10:839*.
94. West, M. A., R. P. Wallin, S. P. Matthews, H. G. Svensson, R. Zaru, H. G. Ljunggren, A. R. Prescott, and C. Watts. 2004. Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science 305:1153*.

95. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 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*.
96. Norbury, C. C., B. J. Chambers, A. R. Prescott, H. G. Ljunggren, and C. Watts. 1997. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur J Immunol 27:280*.
97. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med 189:371*.
98. Basu, S., R. J. Binder, T. Ramalingam, and P. K. Srivastava. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity 14:303*.
99. Shakushiro, K., Y. Yamasaki, M. Nishikawa, and Y. Takakura. 2004. Efficient scavenger receptor-mediated uptake and cross-presentation of negatively charged soluble antigens by dendritic cells. *Immunology 112:211*.
100. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 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*.
101. Figdor, C. G., Y. van Kooyk, and G. J. Adema. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat Rev Immunol 2:77*.
102. Cambi, A., and C. G. Figdor. 2003. Dual function of C-type lectin-like receptors in the immune system. *Curr Opin Cell Biol 15:539*.
103. van Kooyk, Y., and T. B. Geijtenbeek. 2003. DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol 3:697*.
104. McGreal, E. P., J. L. Miller, and S. Gordon. 2005. Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr Opin Immunol 17:18*.
105. Robinson, M. J., D. Sancho, E. C. Slack, S. LeibundGut-Landmann, and C. Reis e Sousa. 2006. Myeloid C-type lectins in innate immunity. *Nat Immunol 7:1258*.
106. Cambi, A., and C. G. Figdor. 2005. Levels of complexity in pathogen recognition by C-type lectins. *Curr Opin Immunol 17:345*.
107. Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, and M. C. Nussenzweig. 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med 194:769*.
108. Geijtenbeek, T. B., S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med 197:7*.
109. Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med 197:1107*.
110. Pyz, E., A. S. Marshall, S. Gordon, and G. D. Brown. 2006. C-type lectin-like receptors on myeloid cells. *Ann Med 38:242*.
111. Taylor, P. R., S. Gordon, and L. Martinez-Pomares. 2005. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol 26:104*.

112. Martinez-Pomares, L., P. R. Crocker, R. Da Silva, N. Holmes, C. Colominas, P. Rudd, R. Dwek, and S. Gordon. 1999. Cell-specific glycoforms of sialoadhesin and CD45 are counter-receptors for the cysteine-rich domain of the mannose receptor. *J Biol Chem 274:35211*.
113. Leteux, C., W. Chai, R. W. Loveless, C. T. Yuen, L. Uhlin-Hansen, Y. Combarnous, M. Jankovic, S. C. Maric, Z. Misulovin, M. C. Nussenzweig, and T. Feizi. 2000. The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and sulfated oligosaccharides of blood group Lewis(a) and Lewis(x) types in addition to the sulfated N-glycans of lutropin. *J Exp Med 191:1117*.
114. Lee, S. J., S. Evers, D. Roeder, A. F. Parlow, J. Risteli, L. Risteli, Y. C. Lee, T. Feizi, H. Langen, and M. C. Nussenzweig. 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science 295:1898*.
115. Mi, Y., S. D. Shapiro, and J. U. Baenziger. 2002. Regulation of lutropin circulatory half-life by the mannose/N-acetylgalactosamine-4-SO4 receptor is critical for implantation in vivo. *J Clin Invest 109:269*.
116. Lee, S. J., N. Y. Zheng, M. Clavijo, and M. C. Nussenzweig. 2003. Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect Immun 71:437*.
117. Swain, S. D., S. J. Lee, M. C. Nussenzweig, and A. G. Harmsen. 2003. Absence of the macrophage mannose receptor in mice does not increase susceptibility to Pneumocystis carinii infection in vivo. *Infect Immun 71:6213*.
118. Imai, K., and T. Yoshimura. 1994. Endocytosis of lysosomal acid phosphatase; involvement of mannose receptor and effect of lectins. *Biochem Mol Biol Int 33:1201*.
119. Shepherd, V. L., and J. R. Hoidal. 1990. Clearance of neutrophil-derived myeloperoxidase by the macrophage mannose receptor. *Am J Respir Cell Mol Biol 2:335*.
120. Irjala, H., E. L. Johansson, R. Grenman, K. Alanen, M. Salmi, and S. Jalkanen. 2001. Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. *J Exp Med 194:1033*.
121. Engering, A. J., M. Cella, D. Fluitsma, M. Brockhaus, E. C. Hoefsmit, A. Lanzavecchia, and J. Pieters. 1997. The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur J Immunol 27:2417*.
122. Ramakrishna, V., J. F. Treml, L. Vitale, J. E. Connolly, T. O'Neill, P. A. Smith, C. L. Jones, L. Z. He, J. Goldstein, P. K. Wallace, T. Keler, and M. J. Endres. 2004. Mannose receptor targeting of tumor antigen pmel17 to human dendritic cells directs anti-melanoma T cell responses via multiple HLA molecules. *J Immunol 172:2845*.
123. Tan, M. C., A. M. Mommaas, J. W. Drijfhout, R. Jordens, J. J. Onderwater, D. Verwoerd, A. A. Mulder, A. N. van der Heiden, D. Scheidegger, L. C. Oomen, T. H. Ottenhoff, A. Tulp, J. J. Neefjes, and F. Koning. 1997. Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur J Immunol 27:2426*.
124. Apostolopoulos, V., N. Barnes, G. A. Pietersz, and I. F. McKenzie. 2000. Ex vivo targeting of the macrophage mannose receptor generates anti-tumor CTL responses. *Vaccine 18:3174*.

125. Kim, T. H., H. Jin, H. W. Kim, M. H. Cho, and C. S. Cho. 2006. Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. *Mol Cancer Ther 5:1723*.
126. Loveland, B. E., A. Zhao, S. White, H. Gan, K. Hamilton, P. X. Xing, G. A. Pietersz, V. Apostolopoulos, H. Vaughan, V. Karanikas, P. Kyriakou, I. F. McKenzie, and P. L. Mitchell. 2006. Mannan-MUC1-pulsed dendritic cell immunotherapy: a phase I trial in patients with adenocarcinoma. *Clin Cancer Res 12:869*.
127. Bernhard, O. K., J. Lai, J. Wilkinson, M. M. Sheil, and A. L. Cunningham. 2004. Proteomic analysis of DC-SIGN on dendritic cells detects tetramers required for ligand binding but no association with CD4. *J Biol Chem 279:51828*.
128. Mahnke, K., M. Guo, S. Lee, H. Sepulveda, S. L. Swain, M. Nussenzweig, and R. M. Steinman. 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*.
129. Prigozy, T. I., P. A. Sieling, D. Clemens, P. L. Stewart, S. M. Behar, S. A. Porcelli, M. B. Brenner, R. L. Modlin, and M. Kronenberg. 1997. The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity 6:187*.
130. East, L., and C. M. Isacke. 2002. The mannose receptor family. *Biochim Biophys Acta 1572:364*.
131. Linehan, S. A., L. Martinez-Pomares, P. D. Stahl, and S. Gordon. 1999. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: In situ expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. *J Exp Med 189:1961*.
132. Martinez-Pomares, L., J. A. Mahoney, R. Kaposzta, S. A. Linehan, P. D. Stahl, and S. Gordon. 1998. A functional soluble form of the murine mannose receptor is produced by macrophages in vitro and is present in mouse serum. *J Biol Chem 273:23376*.
133. Martinez-Pomares, L., M. Kosco-Vilbois, E. Darley, P. Tree, S. Herren, J. Y. Bonnefoy, and S. Gordon. 1996. Fc chimeric protein containing the cysteine-rich domain of the murine mannose receptor binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers. *J Exp Med 184:1927*.
134. Berney, C., S. Herren, C. A. Power, S. Gordon, L. Martinez-Pomares, and M. H. Kosco-Vilbois. 1999. A member of the dendritic cell family that enters B cell follicles and stimulates primary antibody responses identified by a mannose receptor fusion protein. *J Exp Med 190:851*.
135. Yu, P., Y. Wang, R. K. Chin, L. Martinez-Pomares, S. Gordon, M. H. Kosco-Vibois, J. Cyster, and Y. X. Fu. 2002. B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *J Immunol 168:5117*.
136. Nigou, J., C. Zelle-Rieser, M. Gilleron, M. Thurnher, and G. Puzo. 2001. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol 166:7477*.
137. Chieppa, M., G. Bianchi, A. Doni, A. Del Prete, M. Sironi, G. Laskarin, P. Monti, L. Piemonti, A. Biondi, A. Mantovani, M. Introna, and P. Allavena. 2003. Cross-linking

of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol 171:4552*.
138. Pietrella, D., C. Corbucci, S. Perito, G. Bistoni, and A. Vecchiarelli. 2005. Mannoproteins from Cryptococcus neoformans promote dendritic cell maturation and activation. *Infect Immun 73:820*.
139. von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol 3:867*.
140. Randolph, G. J., V. Angeli, and M. A. Swartz. 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol 5:617*.
141. Gretz, J. E., A. O. Anderson, and S. Shaw. 1997. Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex. *Immunol Rev 156:11*.
142. Drumea-Mirancea, M., J. T. Wessels, C. A. Muller, M. Essl, J. A. Eble, E. Tolosa, M. Koch, D. P. Reinhardt, M. Sixt, L. Sorokin, Y. D. Stierhof, H. Schwarz, and G. Klein. 2006. Characterization of a conduit system containing laminin-5 in the human thymus: a potential transport system for small molecules. *J Cell Sci 119:1396*.
143. Nolte, M. A., J. A. Belien, I. Schadee-Eestermans, W. Jansen, W. W. Unger, N. van Rooijen, G. Kraal, and R. E. Mebius. 2003. A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J Exp Med 198:505*.
144. Gretz, J. E., C. C. Norbury, A. O. Anderson, A. E. Proudfoot, and S. Shaw. 2000. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med 192:1425*.
145. Baekkevold, E. S., T. Yamanaka, R. T. Palframan, H. S. Carlsen, F. P. Reinholt, U. H. von Andrian, P. Brandtzaeg, and G. Haraldsen. 2001. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med 193:1105*.
146. Palframan, R. T., S. Jung, G. Cheng, W. Weninger, Y. Luo, M. Dorf, D. R. Littman, B. J. Rollins, H. Zweerink, A. Rot, and U. H. von Andrian. 2001. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med 194:1361*.
147. Pior, J., T. Vogl, C. Sorg, and E. MacHer. 1999. Free hapten molecules are dispersed by way of the bloodstream during contact sensitization to fluorescein isothiocyanate. *J Invest Dermatol 113:888*.
148. Ingulli, E., D. R. Ulman, M. M. Lucido, and M. K. Jenkins. 2002. In situ analysis reveals physical interactions between CD11b+ dendritic cells and antigen-specific CD4 T cells after subcutaneous injection of antigen. *J Immunol 169:2247*.
149. Itano, A. A., S. J. McSorley, R. L. Reinhardt, B. D. Ehst, E. Ingulli, A. Y. Rudensky, and M. K. Jenkins. 2003. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity 19:47*.
150. Sixt, M., N. Kanazawa, M. Selg, T. Samson, G. Roos, D. P. Reinhardt, R. Pabst, M. B. Lutz, and L. Sorokin. 2005. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity 22:19*.
151. Allan, R. S., J. Waithman, S. Bedoui, C. M. Jones, J. A. Villadangos, Y. Zhan, A. M. Lew, K. Shortman, W. R. Heath, and F. R. Carbone. 2006. Migratory dendritic cells

transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity 25:153*.
152. Cavanagh, L. L., and U. H. Von Andrian. 2002. Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol 80:448*.
153. Ohl, L., M. Mohaupt, N. Czeloth, G. Hintzen, Z. Kiafard, J. Zwirner, T. Blankenstein, G. Henning, and R. Forster. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity 21:279*.
154. Jang, M. H., N. Sougawa, T. Tanaka, T. Hirata, T. Hiroi, K. Tohya, Z. Guo, E. Umemoto, Y. Ebisuno, B. G. Yang, J. Y. Seoh, M. Lipp, H. Kiyono, and M. Miyasaka. 2006. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol 176:803*.
155. Bachmann, M. F., M. Kopf, and B. J. Marsland. 2006. Chemokines: more than just road signs. *Nat Rev Immunol 6:159*.
156. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell 99:23*.
157. Randolph, G. J. 2001. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. *Semin Immunol 13:267*.
158. Sanchez-Sanchez, N., L. Riol-Blanco, and J. L. Rodriguez-Fernandez. 2006. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol 176:5153*.
159. Czeloth, N., G. Bernhardt, F. Hofmann, H. Genth, and R. Forster. 2005. Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol 175:2960*.
160. Walker, L. S., and A. K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol 2:11*.
161. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu Rev Immunol 23:515*.
162. Lechner, O., J. Lauber, A. Franzke, A. Sarukhan, H. von Boehmer, and J. Buer. 2001. Fingerprints of anergic T cells. *Curr Biol 11:587*.
163. Hochweller, K., and S. M. Anderton. 2005. Kinetics of costimulatory molecule expression by T cells and dendritic cells during the induction of tolerance versus immunity in vivo. *Eur J Immunol 35:1086*.
164. Heissmeyer, V., F. Macian, S. H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y. C. Liu, M. L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat Immunol 5:255*.
165. Hundt, M., H. Tabata, M. S. Jeon, K. Hayashi, Y. Tanaka, R. Krishna, L. De Giorgio, Y. C. Liu, M. Fukata, and A. Altman. 2006. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. *Immunity 24:513*.
166. Olenchock, B. A., R. Guo, J. H. Carpenter, M. Jordan, M. K. Topham, G. A. Koretzky, and X. P. Zhong. 2006. Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat Immunol 7:1174*.
167. Zha, Y., R. Marks, A. W. Ho, A. C. Peterson, S. Janardhan, I. Brown, K. Praveen, S. Stang, J. C. Stone, and T. F. Gajewski. 2006. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nat Immunol 7:1166*.
168. Ishikawa, S., T. Sato, M. Abe, S. Nagai, N. Onai, H. Yoneyama, Y. Zhang, T. Suzuki, S. Hashimoto, T. Shirai, M. Lipp, and K. Matsushima. 2001. Aberrant high expression

of B lymphocyte chemokine (BLC/CXCL13) by C11b+CD11c+ dendritic cells in murine lupus and preferential chemotaxis of B1 cells towards BLC. *J Exp Med 193:1393*.

169. Liu, Z., and L. Lefrancois. 2004. Intestinal epithelial antigen induces mucosal CD8 T cell tolerance, activation, and inflammatory response. *J Immunol 173:4324*.
170. Redmond, W. L., and L. A. Sherman. 2005. Peripheral tolerance of CD8 T lymphocytes. *Immunity 22:275*.
171. Kurts, C., W. R. Heath, H. Kosaka, J. F. Miller, and F. R. Carbone. 1998. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J Exp Med 188:415*.
172. Davey, G. M., C. Kurts, J. F. Miller, P. Bouillet, A. Strasser, A. G. Brooks, F. R. Carbone, and W. R. Heath. 2002. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J Exp Med 196:947*.
173. Probst, H. C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek. 2005. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol 6:280*.
174. Redmond, W. L., B. C. Marincek, and L. A. Sherman. 2005. Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo. *J Immunol 174:2046*.
175. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol 1:426*.
176. Schluns, K. S., K. Williams, A. Ma, X. X. Zheng, and L. Lefrancois. 2002. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol 168:4827*.
177. Malek, T. R., and A. L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol 4:665*.
178. Lefrancois, L., J. D. Altman, K. Williams, and S. Olson. 2000. Soluble antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. *J Immunol 164:725*.
179. Lee, J. W., M. Epardaud, J. Sun, J. E. Becker, A. C. Cheng, A. R. Yonekura, J. K. Heath, and S. J. Turley. 2007. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol 8:181*.
180. Liblau, R. S., R. Tisch, K. Shokat, X. Yang, N. Dumont, C. C. Goodnow, and H. O. McDevitt. 1996. Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. *Proc Natl Acad Sci U S A 93:3031*.
181. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science 171:813*.
182. Burgdorf, S., V. Lukacs-Kornek, and C. Kurts. 2006. The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J Immunol 176:6770*.
183. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. In *J Exp Med*, Vol. 184, p. 923.

184. Allan, R. S., C. M. Smith, G. T. Belz, A. L. van Lint, L. M. Wakim, W. R. Heath, and F. R. Carbone. 2003. Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science 301:1925*.
185. Dudziak, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, R. M. Steinman, and M. C. Nussenzweig. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science 315:107*.
186. Qi, Z., I. Whitt, A. Mehta, J. Jin, M. Zhao, R. C. Harris, A. B. Fogo, and M. D. Breyer. 2004. Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *Am J Physiol Renal Physiol 286:F590*.
187. Lutz, M. B., P. Rovere, M. J. Kleijmeer, M. Rescigno, C. U. Assmann, V. M. Oorschot, H. J. Geuze, J. Trucy, D. Demandolx, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J Immunol 159:3707*.
188. Gutgemann, I., J. M. Darling, H. B. Greenberg, M. M. Davis, and Y. H. Chien. 2002. A blood-borne antigen induces rapid T-B cell contact: a potential mechanism for tolerance induction. *Immunology 107:420*.
189. Brown, I. E., C. Blank, J. Kline, A. K. Kacha, and T. F. Gajewski. 2006. Homeostatic proliferation as an isolated variable reverses CD8+ T cell anergy and promotes tumor rejection. *J Immunol 177:4521*.
190. Kamath, A. T., J. Pooley, M. A. O'Keeffe, D. Vremec, Y. Zhan, A. M. Lew, A. D'Amico, L. Wu, D. F. Tough, and K. Shortman. 2000. The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J Immunol 165:6762*.
191. McIntosh, G. H., and B. Morris. 1971. The lymphatics of the kidney and the formation of renal lymph. *J Physiol 214:365*.
192. Matsui, K., K. Nagy-Bojarsky, P. Laakkonen, S. Krieger, K. Mechtler, S. Uchida, S. Geleff, D. H. Kang, R. J. Johnson, and D. Kerjaschki. 2003. Lymphatic microvessels in the rat remnant kidney model of renal fibrosis: aminopeptidase p and podoplanin are discriminatory markers for endothelial cells of blood and lymphatic vessels. *J Am Soc Nephrol 14:1981*.
193. Kerjaschki, D., H. M. Regele, I. Moosberger, K. Nagy-Bojarski, B. Watschinger, A. Soleiman, P. Birner, S. Krieger, A. Hovorka, G. Silberhumer, P. Laakkonen, T. Petrova, B. Langer, and I. Raab. 2004. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol 15:603*.

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11. Curriculum vitae

Dr.med (HU) Veronika Lukacs-Kornek

Personal Data

Date of birth:	XX.XX.XXXX
Place of birth:	XXXXX
Nationality:	XXXXX
Marital status:	married
Address:	XXXXX

Education

1983-1991	Primary School in Banreve, Hungary
1991-1995	Attila Jozsef Gymnasium, Ozd Hungary
1995	Abitur
1995-2001	Semmelweis University of Medicine in Budapest
1998-2000	Working as volunteer demonstrator at Semmelweis University in Budapest in the Institute of Genetic, Cell and Immunobiology
1999	Rectors Competition of Medical University of Budapest First Prize Subtitle: The role of IL-6 and sIL-6R in SLE Supervisor : Dr Sara Toth
1997-2001	Member of the Union of Research Students of the Semmelweis University
2001	Medical degree (*summa cum laude*)

	Medical Doctor Thesis: Physiological and pathological aspects of cytokines Supervisor : Dr. Sara Toth
2001-2002	Institute of Biochemistry II. University of Cologne Medical Faculty
2002	DAAD fellowship
2003 August-2007 March	Promotion Institute of Molecular Medicine and Experimental Immunology Supervisor: Prof. Christian Kurts

11.1 Publications

Veronika Lukacs-Kornek*, Sven Burgdorf*, Christian Kurts
The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. **J. Immunol. 2006 176(11): 6770-6.** * Authors contributed equally to this work

Veronika Lukacs-Kornek, Sven Burgdorf, Sabine Specht, Miroslaw Kornek, Christian Kurts. Induction of $CD8^+$ T cell cross-tolerance by lymph node-resident dendritic cells **(in review)**

11.2 Oral presentations

Veronika Lukacs-Kornek, Juliane Scholz, Dirk Benke und Christian Kurts. Soluble small molecular weight antigens are selectively taken up and cross-presented by kidney dendritic. **Immunology Summer-School 2006 Sardinia**

Veronika Lukacs-Kornek, Sven Burgdorf, Sabine Specht, Miroslaw Kornek, Christian Kurts The kidney concentrates blood-borne soluble antigens and dispatches them to the renal lymph node for tolerization of $CD8^+$ T cells. **19th Meeting of the ERCSG (European Renal Cell Study Group) 2007 Paris**

11.3 Posters

Veronika Lukacs-Kornek, Juliane Scholz, Dirk Benke and Christian Kurts. Cell-independent transport of antigens filtered in the kidney glomeruli to the renal lymph node. **16th European Congress of Immunology, 2006 Paris**

Sven Burgdorf, **Veronika Lukacs-Kornek**, Andreas Kautz, Christian Kurts. Influence of the mechanism of antigen uptake on MHC I verus MHC II-restricted antigen presentation. **16th European Congress of Immunology, 2006 Paris**

The Mannose Receptor Mediates Uptake of Soluble but Not of Cell-Associated Antigen for Cross-Presentation[1]

Sven Burgdorf,[2] Veronika Lukacs-Kornek,[2] and Christian Kurts[3]

The mannose receptor (MR) has been implicated in the recognition and clearance of microorganisms and serum glycoproteins. Its endocytic function has been studied extensively using macrophages, although it is expressed by a variety of cell types, including dendritic cells (DC). In this study, we investigated its role in Ag presentation by DC using $MR^{-/-}$ mice. Uptake of the model Ag, soluble OVA, by bone marrow-derived DC and in vitro activation of OVA-specific CD8 T cells (OT-I cells) strictly depended on the MR. In vivo, MR deficiency impaired endocytosis of soluble OVA by DC and concomitant OT-I cell activation. No alterations in the DC subtype composition in $MR^{-/-}$ mice were accountable. Uptake of cell-associated OVA was unaffected by MR deficiency, resulting in unchanged activation of OT-I cells. These findings demonstrate that DC use the MR for endocytosis of a particular Ag type intended for cross-presentation. *The Journal of Immunology,* **2006, 176: 6770–6776.**

The mannose receptor (MR)[4] is a 180-kDa transmembrane C-type lectin that functions as an endocytic receptor. It has been implicated in the recognition of various microorganisms (1). Furthermore, the MR plays a homeostatic role in the clearance of glycoproteins, such as β-glucuronidase and procollagen, which are up-regulated in the blood serum during inflammation (2). The MR consists of an N-terminal cysteine-rich domain, a fibronectin type II repeat domain, eight carbohydrate recognition domains (CRD), a transmembrane domain, and a short intracellular region (3). The cysteine-rich region mediates binding to sulfated sugar moieties, whereas the CRD bind glycoproteins bearing (for instance) terminal mannose, fucose, and, with a lower affinity, glucose residues (4). Most studies addressing the function of the MR have used macrophages (5), which use it for uptake of mannosylated structures such as dextrans (1). In addition to these cells, the MR has also been detected in liver endothelial cells, dermal microvascular endothelial cells, monocytes, Langerhans cells, and dendritic cells (DC) (6).

DC play a central role in the induction of adaptive immune responses (7). After capturing and internalizing Ag in peripheral organs, they migrate toward the draining lymph nodes, where they can activate naive T cells. For activation of $CD8^+$ T cells, captured extracellular Ag are presented on MHC class I molecules (8)—a process termed cross-presentation—which contributes to the induction of cytotoxicity against many viruses and tumors (9). The murine DC subpopulation expressing the CD8α homodimer has been shown to be particularly relevant for cross-presentation of foreign Ag (10) and of self Ag under homeostatic conditions (11, 12). In the presence of inflammatory stimuli, also CD8α-deficient DC were able to cross-present (13, 14).

Several receptors mediating Ag uptake in DC have been identified, such as Fc-receptors, DC-SIGN and DEC205. A role of the MR in Ag uptake and presentation by DC has been proposed based on the finding that mannosylated proteins are presented more efficiently than nonmannosylated ones (15, 16). It is unclear, however, whether this uptake was due to the MR, because DC express other receptors, such as DC-SIGN, with affinity for mannosylated proteins (6, 17). For the same reason, mannan, a polymer of mannose, which competitively blocks endocytosis of mannose-rich structures, cannot be considered a specific inhibitor of the MR. To overcome these limitations, we have used $MR^{-/-}$ mice to elucidate the role of the MR in the uptake and presentation of soluble vs cell-associated OVA.

Materials and Methods

Mice

$MR^{-/-}$ mice on a C57BL/6 (B6) background were generated and provided by Dr. M. C. Nussenzweig (Rockefeller University, New York, NY) (2). B6 mice bearing the K^b mutant bm1 (bm1 mice) and OT-I Rag-$1^{-/-}$ mice on a B6 background were provided by Dr. W. R. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (18). For all experiments, mice between 8 and 16 wk of age bred under specific pathogen-free conditions were used in accordance with local animal experimentation guidelines.

Abs and reagents

All mAb used were purchased from BD Biosciences, except anti-MR Ab (Serotec) and SF1, which were purified from hybridoma supernatant (American Type Culture Collection) and used after conjugation with alexa$_{488}$. All reagents, if not specified otherwise, were obtained from Sigma-Aldrich.

Generation of bone marrow-derived dendritic cells (BMDC)

BMDC were generated using GM-CSF as described previously (19). At day 7, $CD11c^+$ cells isolated by magnetic separation with the autoMACS system (Miltenyi Biotec) were used for all in vitro experiments. Purity of $CD11c^+$ cells was typically higher than 98%.

Preparation of fluorescent soluble and cell-associated OVA

Soluble OVA was conjugated to a fluorochrome using an alexa$_{647}$ labeling kit (Invitrogen Life Technologies) according to the manufacturer's guidelines. The labeling procedure involved gel filtration as a final step for

Institute of Molecular Medicine and Experimental Immunology (IMMEI), Friedrich-Wilhelms-Universität, Bonn, Germany

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[2] S.B. and V.L-K. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Christian Kurts, Institute of Molecular Medicine and Experimental Immunology, Friedrich-Wilhelms-Universität, 53105 Bonn, Germany. E-mail address: ckurts@web.de

[4] Abbreviations used in this paper: MR, mannose receptor; DC, dendritic cell; OT-I cell, OVA-specific CD8 T cell; CRD, carbohydrate recognition domain; bm1 mice, C57BL/6 mice bearing the K^b mutant bm1; BMDC, bone marrow-derived DC; cLN, cutaneous lymph node; MFI, mean fluorescence intensity.

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removal of low molecular mass molecules such as unbound fluorochrome. For cell-associated OVA, splenocytes from bm1 mice (2×10^8 cells/ml) were incubated with 10 mg/ml OVA-FITC for 10 min at 37°C, UV-irradiated with 15 mJ for 5 min, and washed extensively. For cell culture experiments, 10^6 splenocytes were coincubated with 4×10^5 BMDC for 18 h.

Cross-presentation assays

OT-I cells were isolated from OT-I rag$^{-/-}$ mice as previously described (18) and further purified by a nanobead-based CD8 T cell isolation kit (Miltenyi Biotec). Purity was typically higher than 96% of viable cells; contaminating CD11c$^+$ cells were typically rarer than 0.2%, NK1.1$^+$ cells rarer than 0.03%, and CD4$^+$ cells below 1%. For presentation of soluble OVA, 4×10^5 BMDC were stimulated with 10 μg/ml LPS for 2 h and incubated with 500 μg/ml OVA or 20 nM SIINFEKL (OVA peptide). After another 3 h, cells were washed, fixed with 0.008% glutaraldehyde for 3 min, and coincubated with 2×10^5 OT-I cells. IL-2 concentrations were determined after 18 h by ELISA. For the analysis of cell-associated Ag, bm1 splenocytes were coated with OVA as described above. Splenocytes (10^6) were coincubated with 4×10^5 BMDC and 2×10^5 OT-I cells. IL-2 concentrations in the supernatant were determined by ELISA after 40 h.

Isolation of DC from experimental animals

Cells were isolated from spleen and cutaneous lymph node (cLN) as described before (12). For the preparations from bone marrow, cells were collected by flushing femurs and tibias with PBS. CD11c$^+$ cells from all organs were enriched by magnetic separation using MS25 columns (Miltenyi Biotec). Purity was typically higher than 85%.

Flow cytometry, data analysis, and statistics

Flow cytometry was performed on an LSR (BD Biosciences). Dead cells were excluded by Hoechst-33342 dye. Data were analyzed using Flow-Jo software (Tristar), including calculation of division indices, which indicate the average number of cell divisions. Statistical analysis was done using Excel (Microsoft). All experiments reported here have been reproduced at least twice.

Results

The MR is essential for uptake of soluble but not of cell-associated OVA by BMDC

When we studied uptake of OVA in BMDC, we noticed that only some of the CD11c$^+$ cells took up this model Ag in vitro (Fig. 1, *A* and *B*), as others have noted recently (20). We reasoned that this might be explained by selective expression of an endocytic receptor that mediated uptake of OVA. In support of this hypothesis, we observed a close correlation between the uptake of OVA and the extent of MR expression (Fig. 1*A*). To investigate whether this association was due to a functional role of the MR in Ag uptake, we preincubated the DC with mannan, which competitively inhibits MR-mediated endocytosis (21). This agent blocked the uptake of OVA completely (Fig. 1*B*). To exclude an influence of other receptors with affinity to mannan, we performed this experiment also with BMDC from MR$^{-/-}$ mice (2). These cells did not show any uptake (Fig. 1, *B* and *C*), indicating that the MR was indispensable for endocytosis of soluble OVA by BMDC in vitro. Remarkably, under these experimental conditions, no other receptor appeared to compensate even partially for the absence of the MR.

To exclude the possibility that BMDC generated from MR$^{-/-}$ mice differed from those from wild-type mice in terms of subtype composition or maturation status, we phenotypically characterized these DC. Consistent with reports from others (22, 23), BMDC expressed CD11b but not CD8α (Fig. 1*D*). CD11c and CD11b expression was identical in both BMDC populations (Fig. 1*D*), as was constitutive and LPS-induced expression of costimulatory molecules (Fig. 1*E*), suggesting equivalent states of maturation.

Next, we investigated whether the MR was important also for the uptake of cell-associated OVA, using splenocytes loaded with fluorochrome-labeled OVA (Fig. 2*A*). To demonstrate intracellular uptake, loading was performed at 4°C and at 37°C, because intracellular uptake is energy-dependent, as opposed to extracellular coating. Indeed, uptake at 37°C was significantly higher than at 4°C, suggesting that some of the Ag was transported into the spleen cells. We then induced apoptosis by UV irradiation and cocultured these cells with BMDC from MR$^{-/-}$ mice or controls. After 18 h, the uptake of cell-associated OVA by wild-type and MR$^{-/-}$ cells was indistinguishable (Fig. 2*B*), indicating that receptors other than the MR had mediated endocytosis of cell-associated OVA.

The MR is required for cross-presentation of soluble but not of cell-associated OVA in vitro

The requirement of the MR for OVA uptake suggested a role in Ag presentation. To address this hypothesis, we studied cross-presentation of OVA in coculture experiments with BMDC prepared from B6 or MR$^{-/-}$ donor mice and OVA-specific CD8 T cells isolated from transgenic OT-I mice. Their activation was monitored by measuring IL-2 release into the culture supernatant. This release correlated with the Ag amount in a dose-dependent fashion (Fig. 3*A*). To ensure that OT-I cell activation was in fact due to cross-presentation, and not to coating of the BMDC with peptide fragments present in the OVA solution, we performed incubation with soluble OVA in the presence of the proteasome inhibitor MG132, which inhibits intracellular generation of peptides for loading onto MHC class I molecules (24). This inhibitor prevented IL-2 release nearly completely (Fig. 3*A*), indicating that OT-I cells were indeed activated by OVA peptides generated intracellularly. MG132 did not affect OT-I cell activation by BMDC coated with the OVA peptide SIINFEKL, which is recognized by OT-I cells, demonstrating that in this experimental setting, the ability of OT-I cells to produce IL-2 was not compromised (Fig. 3*A*).

When BMDC from MR$^{-/-}$ mice were used, they induced severely reduced IL-2 production by OT-I cells as compared with wild-type cells (Fig. 3*B*), demonstrating that MR-mediated uptake of soluble OVA could provide Ag for cross-presentation. MR-deficient DC externally loaded with SIINFEKL activated OT-I cells equally well as DC from wild-type mice (Fig. 3*B*), indicating that BMDC from MR$^{-/-}$ mice were not compromised in their general ability to activate T cells. Thus, the MR mediated not only uptake of OVA, but also the resulting activation of OT-I cells.

Because the uptake of cell-associated OVA was not reduced in MR-deficient DC, we tested whether OT-I cell activation remained operative as well. To this end, we loaded splenocytes from bm1 mice with OVA. These mice bear a mutant H2-K^b protein that cannot present OVA to OT-I cells (18). After coating with OVA, apoptosis was induced by UV irradiation. These cells were then cocultured with wild-type and MR-deficient BMDC and OT-I cells. No differences in T cell activation between the two DC types could be observed in vitro (Fig. 3*C*), indicating that the MR not only was dispensable for the uptake but also for intracellular processing of cell-associated OVA for cross-presentation.

The MR contributes to in vivo uptake of soluble but not of cell-associated OVA by DC

Next, we decided to investigate the in vivo role of the MR in Ag uptake. To this end, we injected fluorochrome-labeled soluble OVA into B6 mice. Consistent with our in vitro findings, a close correlation between the uptake of soluble OVA and the expression of the MR in DC was found (Fig. 4, *A* and *B*). DC from MR$^{-/-}$ mice showed significant but incomplete reduction of uptake of soluble OVA (Fig. 4, *C* and *D*). This was most evident in the spleen and in the bone marrow, which have been described as locations in which cross-presentation takes place (8, 25). In the

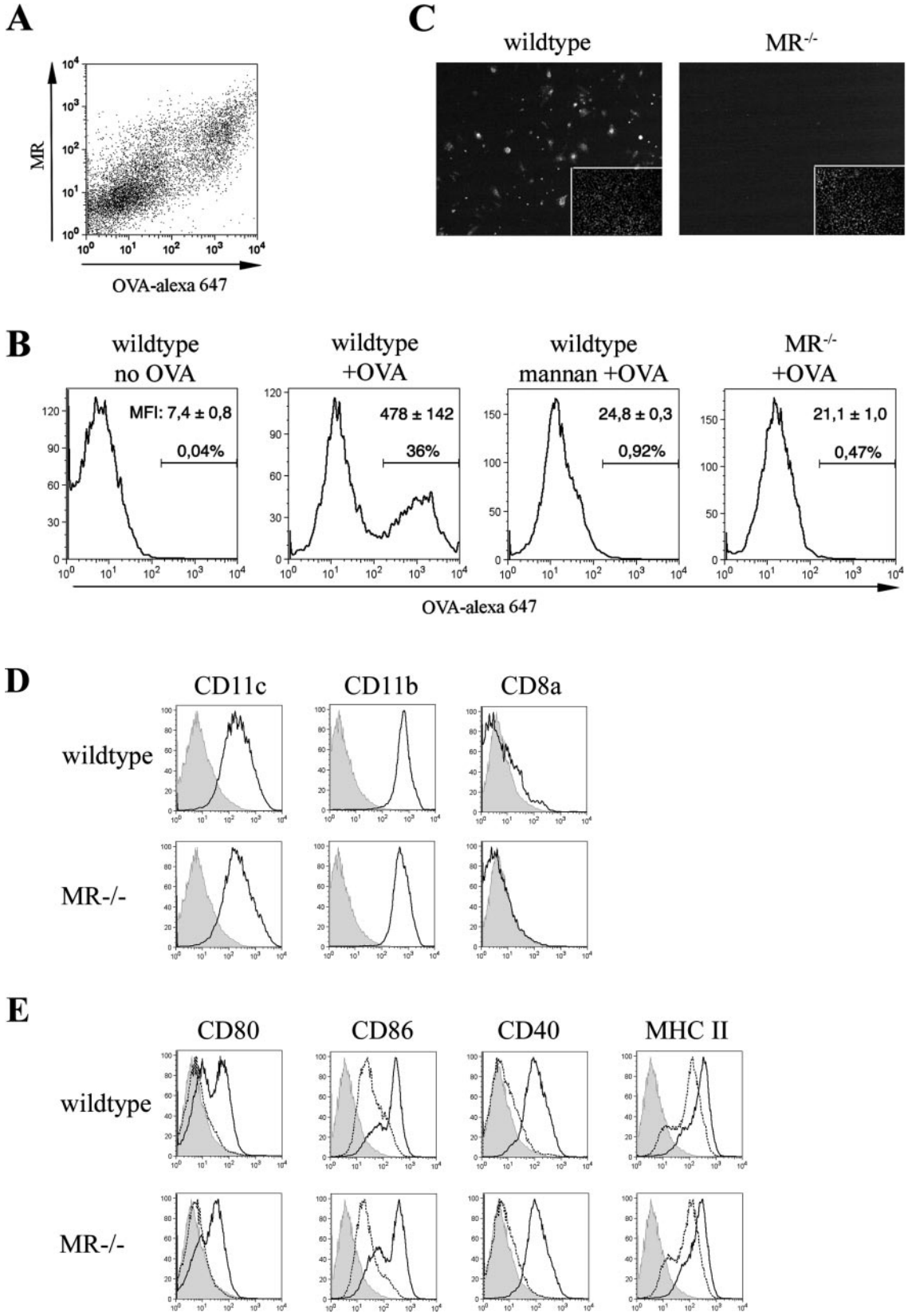

FIGURE 1. The MR is essential for uptake of soluble OVA by BMDC. *A*, CD11c$^+$ BMDC generated from B6 mice were incubated with alexa$_{647}$-conjugated OVA, stained for expression of the MR, and analyzed by flow cytometry. *B*, CD11c$^+$ BMDC from C57/BL6 mice were treated for 30 min with 3 mg/ml mannan and then cocultured for 10 min at 37°C with 10 μg/ml alexa$_{647}$-OVA. Histograms show the uptake profiles of fluorescent Ag by mannan-treated and untreated DC, and by BMDC from MR$^{-/-}$ mice. Numbers indicate the MFI $\pm$ SD; the proportions of the CD11c$^+$ cells that had taken up Ag are given above the histogram area indicators. *C*, Immunofluorescence images visualizing uptake of alexa$_{647}$-OVA by BMDC. *D*, BMDC were prepared from MR$^{-/-}$ and wild-type mice and stained for expression of the subtype markers CD11c, CD11b, and CD8α (solid lines) or control (gray areas). *E*, BMDC from MR$^{-/-}$ and wild-type mice were stained for expression of the maturation markers CD80, CD86, CD40, and MHC class II following stimulation for 24 h with 10 μg/ml LPS (solid lines) or no stimulation (dashed lines).

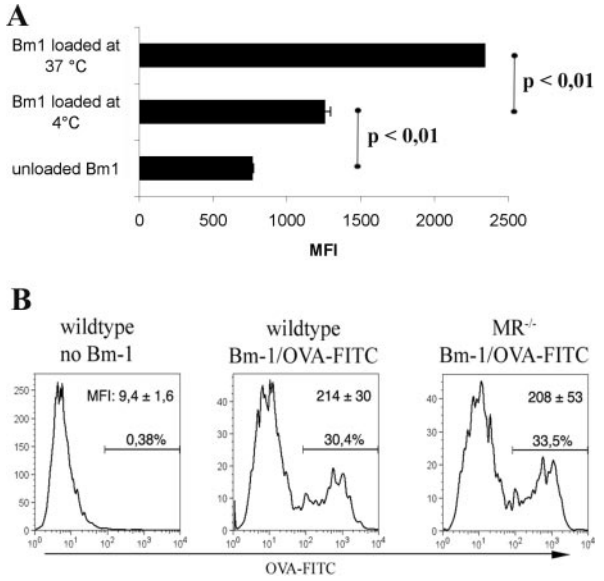

FIGURE 2. The MR is dispensable for uptake of cell-associated OVA by BMDC. *A*, UV-irradiated splenocytes from bm1 mice were incubated for 10 min at 4°C or 37°C with FITC-labeled OVA, and then analyzed for uptake of fluorescent soluble Ag. *B*, UV-irradiated bm1 splenocytes loaded with FITC-OVA at 37°C were cocultured with BMDC from wild-type or $MR^{-/-}$ mice. After 18 h, DC were analyzed for uptake of fluorescent cell-associated Ag. Apoptotic bm1 splenocytes were excluded from analysis by forward scatter/side scatter gating or gating for K^{b+} cells revealed by staining with SF1 Ab. Numbers indicate the MFI ± SD. The proportions of the $CD11c^+$ cells that had taken up Ag are given above the histogram area indicator.

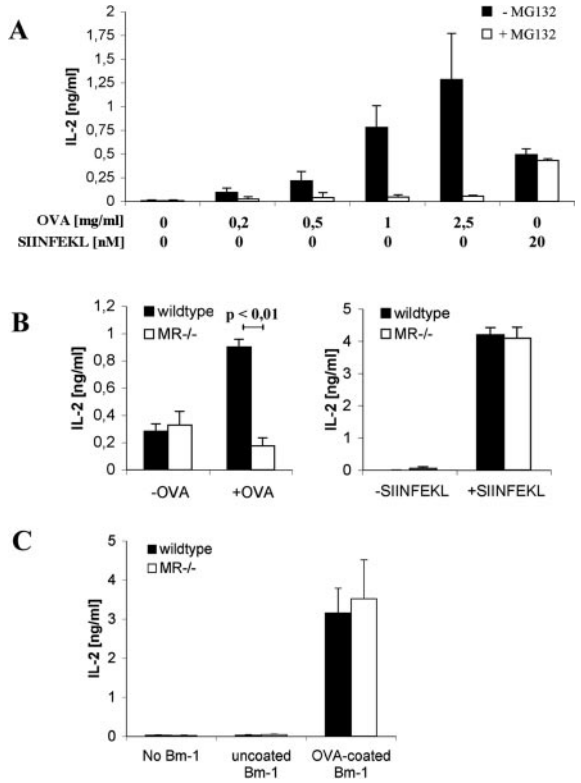

FIGURE 3. The MR is essential for cross-presentation of soluble but not of cell-associated OVA by BMDC to CD8 T cells. *A*, To establish an in vitro system for CD8 T cell activation by cross-presentation, various concentrations of soluble OVA or of the OVA peptide SIINFEKL were coincubated for 2 h with BMDC from B6 mice in the absence (■) or presence (□) of the proteasome inhibitor MG132. DC were then washed, fixed, and cocultured with OT-I cells. After 18 h, IL-2 concentrations in the culture supernatants were determined by ELISA. *B*, BMDC from wild-type (■) or $MR^{-/-}$ (□) B6 mice were incubated with 1 mg/ml OVA or 200 nM SIINFEKL. After an additional 3 h, cells were fixed and naive OT-I cells were added. IL-2 concentrations in the culture supernatants were determined at 18 h after addition of OT-I cells. *C*, Splenocytes from bm1 mice were incubated with OVA, UV irradiated, and then cocultured with BMDC from wild-type or $MR^{-/-}$ mice and OT-I cells. After 40 h, IL-2 concentrations were determined.

cLN, only moderate uptake was observed, which was further reduced in $MR^{-/-}$ mice, albeit not significantly (Fig. 4, *C* and *D*). This reduction was not due to changes of the DC subpopulations present in $MR^{-/-}$ mice, because these were indistinguishable from those in wild-type mice (Fig. 4*E*). These findings demonstrated that the MR was involved also in the in vivo uptake of soluble OVA, but it was not essential, as opposed to its role in vitro. Indeed, in wild-type mice we found some DC that had taken up OVA but did not express the MR (Fig. 4*B*). The mechanisms that partially compensated for the absence of the MR in vivo remain to be identified.

Next, we examined the effect of the MR on the in vivo uptake of cell-associated OVA by injection of UV-irradiated bm1 splenocytes loaded with FITC-labeled OVA into $MR^{-/-}$ mice and controls. DC from the spleen and from the bone marrow of C57/BL6 mice showed significant uptake (Fig. 5, *A* and *B*). The cLN showed only marginal uptake of cell-associated Ag, consistent with reports by others (26). In $MR^{-/-}$ mice, uptake was unaltered (Fig. 5, *A* and *B*), implying that cell-associated Ag had been internalized by mechanisms other than MR-mediated endocytosis.

The MR mediates cross-presentation of soluble but not of cell-associated OVA in vivo

To assess the in vivo role of the MR for CD8 T cell activation by cross-presentation, we injected CFSE-labeled OT-I transgenic T cells into wild-type or MR-deficient recipient mice. After priming with soluble OVA, we analyzed the proliferation of OT-I cells in spleen, bone marrow, and cLN. In $MR^{-/-}$ mice, proliferation of these T cells was substantially reduced (Fig. 6*A*). Also, CD69 expression on OT-I cells was substantially diminished on day 1 in all organs tested in $MR^{-/-}$ mice (data not shown), demonstrating that the diminished proliferation of OT-I cells was due to decreased activation at the priming site, rather than to reduced recirculation of OT-I cells activated elsewhere.

Finally, we examined the in vivo role of the MR in the presentation of cell-associated OVA. Wild-type and $MR^{-/-}$ mice were injected with OT-I cells and primed with OVA-coated, UV-irradiated bm1 splenocytes. Proliferation of OT-I cells in the spleen and in the bone marrow was not reduced in $MR^{-/-}$ mice (Fig. 6*B*). Moreover, no alterations in up-regulation of CD69 in the activated T cells were observed (data not shown). Consistent with the marginal Ag uptake in the cLN, no proliferation of OT-I cells was observed in this location (Fig. 6*B*). Thus, mechanisms other than MR-mediated Ag uptake were responsible for CD8 T cell activation by cross-presentation of cell-associated Ag.

Discussion

The molecular mechanisms that mediate Ag uptake for cross-presentation are unresolved. The present study is the first to identify a receptor involved in this process, by demonstrating that Ag endocytosed via the MR gained access to the cross-presentation pathway. We showed that the MR was important for the uptake of

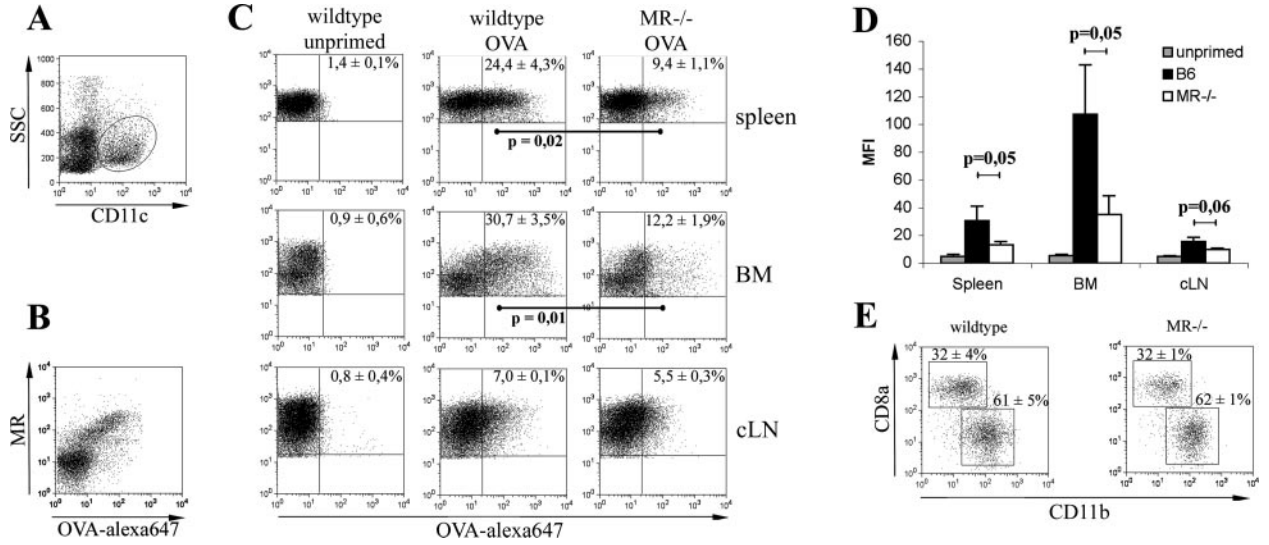

FIGURE 4. The MR predominantly mediates in vivo uptake of soluble OVA. *A*, Gating scheme used for analysis of Ag uptake by splenic DC. *B*, B6 mice were i.v. injected with alexa$_{647}$-conjugated OVA (5 μg/g body weight). After 45 min, single-cell suspensions from the spleen were stained for CD11c and MR expression, and analyzed using the gate shown in *A*. *C*, Wild-type and MR-deficient C57/BL6 mice were i.v. injected with 5 μg/g body weight alexa$_{647}$-conjugated OVA. After 45 min, DC were isolated from spleen, bone marrow, and cLN, and analyzed by flow cytometry. Numbers give the proportions of alexa$_{647}^{+}$ cells ± SD within the viable CD11c^{+} cells (n = 3 or 4 mice). *D*, MFI ± SD of the cells from *C*. *E*, Alexa$_{647}^{+}$ splenic CD11c^{+} cells were stained for expression of the DC subtype markers CD11b and CD8α, and analyzed by flow cytometry.

soluble OVA by DC in vivo, and that it was even essential for BMDC in vitro. Concomitantly, the activation of naive CD8 T cells by soluble OVA was diminished and in fact abrogated when BMDC were used. This was most likely due to reduced cross-presentation of OVA, because the absence of the MR did not affect expression of costimulatory molecules. Furthermore, MR-deficient DC loaded with OVA peptide induced a T cell response equal to that induced by wild-type DC, implying that the costimulatory signals provided must have been similar. Also, differences in the DC subtype composition of MR-deficient mice were unchanged, in particular the content of CD8α^{+} DC, which have been reported to mediate in vivo cross-presentation of soluble and cell associated OVA (10, 14).

As opposed to the clear-cut in vitro situation, uptake and cross-presentation of soluble OVA in vivo was not completely abolished in MR$^{-/-}$ mice, yet it was significantly reduced. This may indicate that BMDC do not mimic all mechanisms involved in Ag uptake by DC in vivo. These mechanisms may involve the generation of OVA peptides by endogenous proteases, or Ag uptake by additional cellular receptors, such as the lectins DC-SIGN and DEC205, whose expression might differ between BMDC and DC in living animals (17, 27). This may be the case for CD8α^{+} DC that were shown to mediate cross-presentation in vivo (10, 14), because these are known to be absent from BMDC generated by standard methodology (22).

Uptake of cell-associated OVA and the resulting activation of

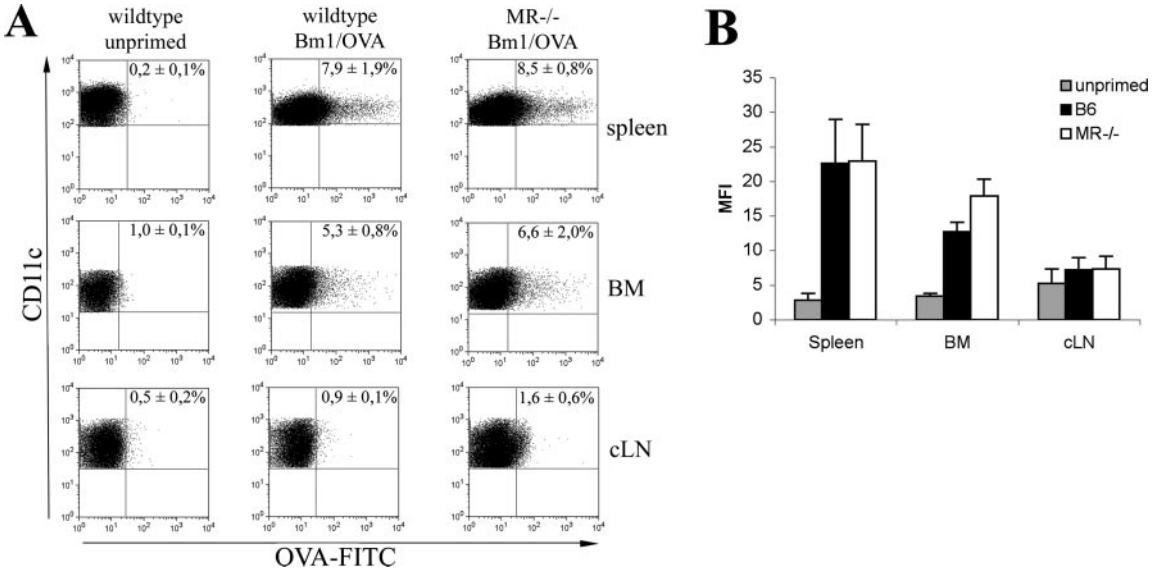

FIGURE 5. The MR is dispensable for in vivo uptake of cell-associated OVA. *A*, FITC-OVA-loaded bm1 cells (20×10^{6}) were UV-irradiated and injected i.v. into wild-type and MR-deficient B6 mice. After 12 h, DC were isolated from spleen, bone marrow, and cLN, and the proportion of alexa$_{647}^{+}$ cells in the viable CD11c^{+} cells was determined (n = 3 or 4 mice). *B*, MFI ± SD of the cells from *A*.

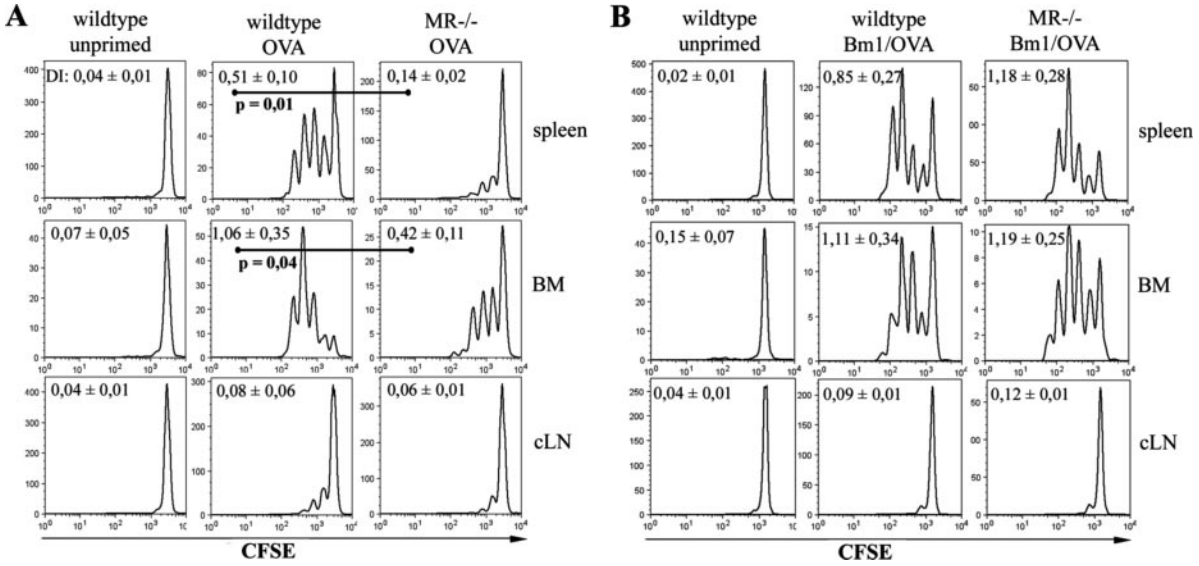

FIGURE 6. The MR contributes to in vivo CD8 T cell activation by cross-presentation of soluble but not of cell-associated OVA. *A*, CFSE-labeled OT-I cells (2×10^6) were injected into wild-type or $MR^{-/-}$ B6 mice. After 18 h, 5 μg OVA/g body weight was injected i.v. After an additional 40 h, the proliferation of OT-I cells was analyzed in the spleen, bone marrow, and cLN. Shown are the $CFSE^+$ $CD8^+$ cells present in the organs. *B*, Same experimental setup as in *A*, except that 20×10^6 OVA-loaded, UV-irradiated bm1 splenocytes were used instead of soluble OVA. Numbers give the division index $\pm$ SD ($n = 3$ or 4 mice).

OT-I cells were not impaired by the absence of the MR, either in vivo or in vitro. The cell-associated Ag used in the present study was located intracellularly, at least in part. Such Ag may be available for endocytosis by one or several distinct receptors. Reports implicating CD36 in uptake of Ag borne by apoptotic cells are controversial (28). Thus, the mechanisms mediating Ag uptake for classical cross-priming, as described by Bevan (8), remain to be resolved. Nevertheless, our findings demonstrated that additional pathways exist for cross-presentation of Ag carried by microorganisms, or of viral Ag within infected host cells.

The MR is mostly known for its role as a scavenger receptor of macrophages. A role in Ag presentation has been proposed by others based on its expression by DC and on its role in the uptake of mannosylated structures such as dextrans or horseradish peroxidase in a mannan-blockable fashion into cellular compartments that contained MHC class II molecules (21). Such uptake could result in stimulation of CD4 T cell clones in vitro (16), suggesting a role for the MR in adaptive immunity. The findings reported here support such a role by demonstrating MR-mediated activation of naive CD8 T cells, and by providing evidence for in vivo relevance of such activation. Furthermore, the use of $MR^{-/-}$ mice in the present study precluded the specificity concerns that apply to mannan blockade of the MR (6).

Our findings suggest that targeting of the MR may be useful for introducing extracellular Ag into the MHC class I-restricted Ag presentation pathway. Targeting of a related lectin, DEC205, has been shown to result in tolerogenic CD4 and CD8 T cell activation (27). It will be interesting to investigate whether Ag uptake by the MR would result in immunogenic or tolerogenic CTL priming, or both, depending on the absence or presence of inflammatory stimuli. Finally, the indispensability of the MR for uptake of soluble OVA by BMDC may allow further in vitro dissection of intracellular mechanisms governing cross-presentation.

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Disclosures

The authors have no financial conflict of interest.

References

1. McGreal, E. P., J. L. Miller, and S. Gordon. 2005. Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr. Opin. Immunol.* 17: 18–24.
2. Lee, S. J., S. Evers, D. Roeder, A. F. Parlow, J. Risteli, L. Risteli, Y. C. Lee, T. Feizi, H. Langen, and M. C. Nussenzweig. 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science* 295: 1898–1901.
3. Ezekowitz, R. A., K. Sastry, P. Bailly, and A. Warner. 1990. Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. *J. Exp. Med.* 172: 1785–1794.
4. Largent, B. L., K. M. Walton, C. A. Hoppe, Y. C. Lee, and R. L. Schnaar. 1984. Carbohydrate-specific adhesion of alveolar macrophages to mannose-derivatized surfaces. *J. Biol. Chem.* 259: 1764–1769.
5. Harris, N., M. Super, M. Rits, G. Chang, and R. A. Ezekowitz. 1992. Characterization of the murine macrophage mannose receptor: demonstration that the down-regulation of receptor expression mediated by interferon-γ occurs at the level of transcription. *Blood* 80: 2363–2373.
6. Figdor, C. G., Y. van Kooyk, and G. J. Adema. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol* 2: 77–84.
7. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.
8. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143: 1283–1288.
9. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199: 9–26.
10. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. $CD8^+$ but not $CD8^-$ dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192: 1685–1696.
11. Belz, G. T., F. R. Carbone, and W. R. Heath. 2002. Cross-presentation of antigens by dendritic cells. *Crit. Rev. Immunol.* 22: 439–448.
12. Benke, D., T. Krüger, A. Lang, E. E. Hamilton-Williams, and C. Kurts. 2006. Inclusion of brefeldin A during dendritic cell isolation allows in vitro detection of cross-presented self antigens. *J. Immunol. Methods* 310: 12–19.
13. den Haan, J. M., and M. J. Bevan. 2002. Constitutive versus activation-dependent cross-presentation of immune complexes by $CD8^+$ and $CD8^-$ dendritic cells in vivo. *J. Exp. Med.* 196: 817–827.
14. Pooley, J. L., W. R. Heath, and K. Shortman. 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: 5327–5330.

15. Engering, A. J., M. Cella, D. Fluitsma, M. Brockhaus, E. C. Hoefsmit, A. Lanzavecchia, and J. Pieters. 1997. The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur. J. Immunol.* 27: 2417–2425.
16. Tan, M. C., A. M. Mommaas, J. W. Drijfhout, R. Jordens, J. J. Onderwater, D. Verwoerd, A. A. Mulder, A. N. van der Heiden, D. Scheidegger, L. C. Oomen, et al. 1997. Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur. J. Immunol.* 27: 2426–2435.
17. Engering, A., T. B. Geijtenbeek, and Y. van Kooyk. 2002. Immune escape through C-type lectins on dendritic cells. *Trends Immunol.* 23: 480–485.
18. Kurts, C., H. Kosaka, F. R. Carbone, J. F. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive $CD8^+$ T cells. *J. Exp. Med.* 186: 239–245.
19. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
20. Menges, M., T. Baumeister, S. Rossner, P. Stoitzner, N. Romani, A. Gessner, and M. B. Lutz. 2005. IL-4 supports the generation of a dendritic cell subset from murine bone marrow with altered endocytosis capacity. *J. Leukocyte Biol.* 77: 535–543.
21. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J. Exp. Med.* 182: 389–400.
22. Naik, S. H., A. I. Proietto, N. S. Wilson, A. Dakic, P. Schnorrer, M. Fuchsberger, M. H. Lahoud, M. O'Keeffe, Q. X. Shao, W. F. Chen, et al. 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: 6592–6597.
23. Hochrein, H., M. O'Keeffe, and H. Wagner. 2002. Human and mouse plasmacytoid dendritic cells. *Hum. Immunol.* 63: 1103–1110.
24. Imai, J., H. Hasegawa, M. Maruya, S. Koyasu, and I. Yahara. 2005. Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. *Int. Immunol.* 17: 45–53.
25. Feuerer, M., P. Beckhove, N. Garbi, Y. Mahnke, A. Limmer, M. Hommel, G. J. Hammerling, B. Kyewski, A. Hamann, V. Umansky, and V. Schirrmacher. 2003. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat. Med.* 9: 1151–1157.
26. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R. M. Steinman, and K. Inaba. 2002. The $CD8^+$ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195: 1289–1302.
27. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 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: 1627–1638.
28. Belz, G. T., D. Vremec, M. Febbraio, L. Corcoran, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. CD36 is differentially expressed by $CD8^+$ splenic dendritic cells but is not required for cross-presentation in vivo. *J. Immunol.* 168: 6066–6070.