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In vitro the mannose receptor (MR) mediates Ag internalization by dendritic cells (DC) and favors the presentation of mannansylated ligands to T cells. However, in vivo MR seems to play a role not in Ag presentation but in the homeostatic clearance of endogenous ligands, which could have the secondary benefit of reducing the levels of endogenous Ag available for presentation to the adaptive immune system. We have now observed that while MR⁺ cells are consistently absent from T cell areas of spleen and mesenteric lymph nodes (LN), peripheral LN of untreated adult mice contain a minor population of MR⁺MHCI⁺ in the paracortex. This novel MR⁺ cell population can be readily identified by flow cytometry and express markers characteristic of DC. Furthermore, these MR⁺ DC-like cells located in T cell areas can be targeted with MR ligands (anti-MR mAb). Numbers of MR⁺MHCI⁺ cells in the paracortex are increased upon stimulation of the innate immune system and, accordingly, the amount of anti-MR mAb reaching MR⁺MHCI⁺ cells in T cell areas is dramatically enhanced under these conditions. Our results indicate that the MR can act as an Ag-acquisition system in a DC subpopulation restricted to lymphoid organs draining the periphery. Moreover, the effect of TLR agonists on the numbers of these MR⁺ DC suggests that the immunogenicity of MR ligands could be under the control of innate stimulation. In accordance with these observations, ligands highly specific for the MR elicit enhanced humoral responses in vivo only when administered in combination with endotoxin. The Journal of Immunology, 2007, 178: 4975–4983.

Dendritic cells (DCs) are professional APCs with crucial roles in the induction and control of tolerance to self-Ags and immunity to pathogen-derived Ags. These unique cells sample Ag constitutively and migrate from the periphery to secondary lymphoid organs (1, 2) where they present processed Ags to T cells. In the absence of infection Ag presentation will result in tolerance, whereas in the presence of microbial signals DC maturation will occur, facilitating the induction of effector responses. The induction of tolerance to self and innocuous foreign Ags may also be influenced by the efficient clearance of self-Ags by macrophages (MΦs), restricting exposure of DCs to Ags.

In vivo DCs are a rare but heterogeneous collection of cells expressing a wide range of germline-encoded pattern recognition receptors. These receptors encompass several families of molecules including TLRs, C-type lectins, and C-type lectin-like receptors expressed both at the cell surface and intracellularly. DCs are able to sense the presence of foreign microbes via these receptors. Multiple signals provided by a pathogen are transduced upon ligand recognition and ultimately govern the course of the effector response toward the invader.

The mannose receptor (MR) is a C-type lectin that provides an efficient cellular internalization system for both endogenous and microbe-derived molecules and has a well-established role in the maintenance of tissue homeostasis as exemplified in studies of MR-deficient mice generated by Lee et al. (3). These mice exhibited defective clearance of neoglycoconjugates and elevated serum levels of multiple lysosomal hydrolases, indicating impaired clearance (3). The MR recognizes sulfated carbohydrates through its cysteine-rich (CR) domain (4, 5), native and denatured collagens through its fibronectin type II domain (6), and oligosaccharides terminating in mannose, fucose, or N-acetyl glucosamine through its C-type lectin-like carbohydrate recognition domains (recently reviewed in Ref. 7).

No expression of the MR has been documented on murine DC populations in vivo; in agreement with its major role in clearance, the MR is present in most tissue MΦs and in hepatic and lymphatic endothelia (8). In humans, the MR has been detected in cells located within the dermis, lamina propria, and T cell areas of the tonsil (9), in inflammatory epidermal DCs from patients with atopic dermatitis (10), and in cells lining venous sinuses in the spleen (11). Evidence for the involvement of the MR in Ag presentation to the acquired immune system is limited and in some
cases contradictory. The MR is expressed by human and murine DCs generated in vitro, human monocyte-derived DCs (moDCs) and mouse bone marrow-derived DCs. Uptake of mannosylated ligands by moDCs leads to the delivery of Ag to MHCII+ (12) and CD1b+ (13) compartments and enhanced presentation to T cells (14–16). Delivery of the melanoma Ag, pme17 through the MR in human moDCs using an anti-MrnAb-pme17 fusion protein led to Ag presentation via both HLA I and HLA II molecules (17), indicating that in human DCs the MR could provide an efficient mechanism for Ag acquisition and delivery into Ag processing pathways. In mice, bone marrow-derived DCs were shown to internalize Ag through the MR for presentation to T cells, although MR ligands were not presented as efficiently as ligands for DEC-205, another member of the MR family of proteins (18), and MR expression is required for cross-presentation of the soluble model Ag OVA (19). In contrast, Napper and Taylor recently reported that fibroblasts cotransfected with the MR and MHCII were not able to enhance the presentation of glycosylated Ag to T cells (20), and Ags engineered in fungi to enhance mannosylation elicit T cell responses independently of the MR (21).

Several of the endogenous molecules recognized by the MR are targeted by the immune system in autoimmune diseases such as thyroiditis (thyroglobulin) (22, 23), antineutrophil cytoplasm Ab-associated vasculitis (myeloperoxidase) (Ref. 24 and our own unpublished data), rheumatoid arthritis (collagen II, a major component of cartilage) (25), and Goodpasture’s disease (collagen IV) (26). This correlation led us to consider that if the MR contributed to Ag presentation in vivo it could mediate the inappropriate presentation of its endogenous ligands to the acquired immune system. The aim of this work was to investigate whether the MR could mediate Ag acquisition for presentation to the adaptive immune system under any circumstance in vivo. For this purpose we have analyzed MR expression in DC, determined the fate of MR ligands upon in vivo administration and quantified the humoral responses against MR ligands in naive and stimulated animals.

Our results demonstrate that stimulation of the innate immune response has a profound effect on the involvement of MR in the induction of adaptive immune responses. We have identified a novel DC population expressing a functional MR. These MR+ DCs are restricted to peripheral (p) lymph nodes (LNs) and their numbers are controlled by the presence of selected TLR agonists. In agreement with these data, the induction of humoral responses independently of the MR (21).

### Materials and Methods

#### Animals

Mice used in this study (BALB/c, C57BL/6, and MR−/−, which are on the C57BL/6 genetic background) were bred within our own institutional colonies, sex matched, and between the ages of 7 and 16 wk at the time of study. Animals were kept and handled in accordance with institutional guidelines. MR−/− mice were provided by Prof. M. Nussenzweig, (Rockefeller University, New York).

#### Reagents

The TLR agonists used in these studies are LPS purified from the Hae-mophilus influenzae type b strain Eagen (a gift from S. Zamze, Edward Jenner Institute for Vaccine Research, Compton, U.K.); flagellin purified from Salmonella typhimurium (InvivoGen); polyinosinic-polycytidylic acid (poly(I:C); Amersham Biosciences) and (S)-2R-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH, HCl (Pam3CSK4; Calbiochem).

### Tissue digestion

Peripheral (cervical, brachial, axillary, inguinal, and popliteal) and mesenteric LNs were digested with 1.5 mg/ml collagenase D (Roche) and 1 mg/ml DNase I (Roche) in RPMI 1640 for 25 min at 37°C with gentle shaking. Tissues were further broken down with gentle pipetting. Cell suspensions were washed twice in PBS containing 0.5% BSA and 5 mM EDTA. In some experiments cell suspensions were enriched in CD11c+ cells using anti-CD11c MACS beads (Miltenyi Biotec) following the manufacturer’s instructions, and in other experiments cells were used directly for FACs staining.

#### Flow cytometry

Single cell suspensions were blocked for 45 min at 4°C in 5% (v/v) heat-inactivated rabbit serum, 0.5% BSA, 5 mM EDTA, 2 mM NaN3, and 4 mg/ml poly(I:C); Amersham Biosciences) and (S)-2,3-bis(palmitoyloxy)propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH, HCl (Pam3CSK4; Calbiochem). This correlation led us to consider that if the MR contributed to Ag presentation in vivo it could mediate the inappropriate presentation of its endogenous ligands to the acquired immune system. The aim of this work was to investigate whether the MR could mediate Ag acquisition for presentation to the adaptive immune system under any circumstance in vivo. For this purpose we have analyzed MR expression in DC, determined the fate of MR ligands upon in vivo administration and quantified the humoral responses against MR ligands in naive and stimulated animals.

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#### Immunohistochemistry

Slides were fixed for 10 min on ice with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and then blocked with 5% (v/v) normal goat serum (Innolife Technologies) in PBS for 30 min to block irrelevant binding sites. Further blocking of endogenous biotin was achieved using an avidin/biotin blocking kit (Vector Laboratories) as per the manufacturer’s recommendations. Staining Abs were prepared to appropriate concentrations in 5% (v/v) normal goat serum in PBS and incubated with slides for 60 min. Goat anti-rat IgG-Alexa Fluor 488 secondary Ab (Molecular Probes) diluted in PBS was applied for a further 30 min. In the case of double labeling with another Ab also raised in rat, an additional 30 min blocking step was conducted with 100 µg/ml rat IgG (Sigma-Aldrich) before incubation with the secondary biotinylated primary reagent for 60 min. This was followed by 30 min of incubation with a streptavidin–Cy3–Cy5 (Jackson Immunoresearch) or Alexa Fluor 488 (Molecular Probes) secondary reagent. Slides were counterstained with 400 mg/ml 4’,6’-diamidino-2-phenylindole (Sigma-Aldrich) before mounting. Slides were washed between each step with PBS.

#### Ear skin explant

Ears from BALB/c mice were removed at the base and split into dorsal and ventral sides. Each half was placed dermal side down into a well of a 24-well tissue culture plate containing 2 ml of medium and incubated for 24 h in a 5% CO2, 95% humidity incubator at 37°C. Migrated cells were then collected and washed in medium and cytospins were prepared.

In vivo targeting of MR ligands

Anti-MR mAbs or isotype controls were injected s.c. into the forelimb just above the wrist or in some experiments in the leg just above the ankle under anesthesia with 3% isoflurane in air. At various times after injection, the spleen and the peripheral and mesenteric LNs were collected and fresh frozen for immunohistochemical analysis. Injected mAb could be detected in tissue sections by fluorescently labeled goat anti-rat IgG reagents (Molecular Probes/Innolife Technologies).

#### Immunizations

Animals were anesthetized as previously indicated before the s.c. injection of Ag into forelimbs just above the wrist. MR53C, MR53D, and control IgG2a (clone CLIII/10; provided by Dr. R. Hodes, National Institutes of Health) were injected in the mid-forearm (2,442 µl) to saturate the mesenteric LNs. After 7–14 days, animals were sacrificed and bled and sera were prepared and stored at −20°C. The mAbs were purified from hybridoma supernatants prepared in Iscove’s medium containing low endotoxin

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and IgG-depleted FCS (Invitrogen Life Technologies) using a GammaBind Plus Sepharose column (Invitrogen Life Technologies). All preparations were quantified using a BCA assay (Perbio), analyzed for purity by Coomassie staining, and tested for endotoxin contamination using the Limulus amebocyte Lysate assay (Cambrex/BioWhittaker). All proteins were aliquoted and stored at -20°C until required.

**ELISA**

Total mouse anti-rat IgG produced by each animal was determined by ELISA. Flat-bottom, 96-well, high-binding enzyme immunoassay/radioimmunoassay (ELA/RIA) plates (Corning) were coated with 10 µg/ml rat IgG (Sigma-Aldrich) at 50 µl/well in PBS overnight at 4°C. Plates were blocked with 3% BSA (w/v) in PBS for 60 min at 37°C before the addition of appropriate dilutions of serum in duplicate for 1 h at room temperature. Wells were then incubated for 1 h with 50 µl/well anti-mouse IgG-alkaline phosphatase (AP) (Sigma-Aldrich) to detect mouse IgG bound to anti-rat Abs or with anti-mouse IgG1-AP or anti-mouse IgG2a-AP (both BD Pharmingen) to detect specific subclasses. All AP conjugates were used at a 1/1000 dilution in PBS. Absorbance was measured at a wavelength of 405 nm using a microplate reader. Plates were washed three times between incubations in PBS supplemented with 0.1% Tween 20 (Sigma-Aldrich). IgG titers were ascertained by calculating the dilution of serum required to achieve an absorbance value of 0.2. Animals that did not make a detectable IgG response were assigned an arbitrary value, the minimum dilution level of serum used and, thus, the level of detection.

**Statistical analysis**

Statistical analysis was performed using ANOVA and the Bonferroni test with GraphPad Prism software version 3.02. Where appropriate, statistical analysis was performed using ANOVA and the Bonferroni test.

**Results**

**MR** 

**+** cells are found in the outer paracortical areas of selected secondary lymphoid tissues

The expression of the MR in secondary lymphoid tissues was investigated by immunofluorescent staining. In line with our previous studies, MR is abundantly expressed in the medullary regions of LN and the red pulp of the spleen, and small numbers of MR cells were observed in the outer paracortex of LNs close to B cell areas (8). When different LNs were compared we found that the paracortical MR cells were restricted to pLNs (Fig. 1A, left panel), which drain the skin, and absent from mesenteric (m)LNs (Fig. 1A, right panel), which drain the gut. As reported, MR is absent from the white pulp of the spleen (data not shown and Fig. 4A). Further analysis confirmed that paracortical MR+ cells also express MHCII (Fig. 1B), indicating that the MR could be expressed by a subset of DCs.

**Characterization of MR** 

**+** cells in lymph nodes by flow cytometry

To characterize the MR+ MHCII+ cells detected in pLNs, we performed flow cytometric analysis of single cell suspensions prepared from pLNs and mLNs using collagenase digestion as described in Materials and Methods. Cells with high forward and side scatter were gated (these parameters encompass CD11c+ cells) and subsequent analysis was performed. A population of CD11c+MR+ cells was identified in pLN that was absent in mLN (Fig. 2A). To gain a clearer picture of the phenotype of this population, cells were enriched for CD11c and then labeled for MR and MHCII as described in Materials and Methods. An analysis of MR and MHCII expression was performed on the gated CD11c+ cell population. All MR+CD11c+ cells expressed DEC-205+CD11b+CD8αint, whereas MR−CD11c− cells were DEC-205highCD11bhighCD8αlow (where “inter” is intermediate). Different cell preparations were used for each labeling. Quadrants were set according to isotype control labeling.

**Comparison of surface markers expressed by MR**

**+** and MR− CD11c− cells. CD11c− enriched cells from pLN were gated and analyzed for expression of MR and DEC205, CD11b, or CD8α. MR+CD11c+ cells were found to be DEC-205int/hi/CD11bint/CD8αint whereas MR−CD11c− cells were DEC-205high/CD11bhigh/CD8αlow (high) (where “inter” is intermediate). Different cell preparations were used for each labeling. Quadrants were set according to isotype control labeling.
MHCII, with the majority of the cells expressing high levels of MHCII (Fig. 2B). In accordance with previous results, CD11c⁺ MR⁺ MHCII⁺ cells were absent in mLNs. MR⁺CD11c⁻ and MR⁺CD11c⁺ cells from pLN were compared for the expression of several DC-associated Ags (Fig. 3). MR⁺CD11c⁻ cells were found to be DEC-205intCD11bhighCD8αlow, whereas MR⁺CD11c⁺ cells were DEC-205highCD11bintCD8αlow/high (where “int” is intermediate). Based on their levels of DEC-205 and CD11b expression, MR⁺CD11c⁺ cells seemed to correspond to the interstitial DC population described by Henri et al. (27). Further studies showed that MR⁺CD11c⁺ cells expressed the costimulatory molecules CD40 (data not shown) and CD86 (see Fig. 4B).

**Numbers of MR⁺ DC are under the control of innate stimulation**

To address the possibility of MR⁺ DC being influenced by innate stimulation in a similar way to that described for cells expressing ligands for the CR domain of the MR (28–30), we analyzed the effect of systemic and local stimulation with microbial products such as LPS and flagellin on MR⁺ DC numbers by fluorescence microscopy. A prominent increase in MR⁺ cells in the T cell areas of pLN was observed after systemic stimulation with LPS and flagellin (Fig. 4A). Under these conditions, MR⁺ cells in T cell areas were also MHCII⁺ (data not shown). Interestingly, these strong innate stimuli administered through the i.v. route did not alter the absence of MR⁺ DCs in the T cell areas of mLNs or the spleen, further highlighting the restricted anatomical location of MR⁺ DCs. We also observed decreased MR expression in splenic red pulp Mδ after LPS or flagellin treatment. This has previously been shown to occur in response to LPS in vitro (31). Local s.c. administration of LPS (1 μg/site) also induced an increase in paracortical MR⁺ cell numbers in draining LNs (data not shown). The effect of the systemic administration of two other TLR agonists, Pam₃CSK₄, and poly(I:C), on MR⁺ DC numbers was also assessed during this study. A variable increase in MR⁺ DC numbers was observed in pLN 24 h after the i.v. administration of Pam₃CSK₄ (5–20 μg). Interestingly, the i.v. administration of poly(I:C) (5–10 μg) did not induce any increase in MR⁺ DC numbers (data not shown). These data indicate that the number of MR⁺ DCs is...
regulated by selective TLR agonists. These results were supported by the flow cytometric analysis of cells suspensions from the pLNs of LPS-treated or untreated animals, which demonstrated that LPS treatment led to the presence of an increased percentage of CD11c+ cells in pLNs (from 2.15 to 3.3% and from 2.69 to 3.55% in two separate experiments); a higher proportion of MR+MHCII+ and MR+CD86+ cells were detected within the CD11c+ cell population from treated animals (Fig. 4B).

MR+ cells in the skin as potential precursors of MR+ DC in LNs
As shown in Fig. 3, MR+ DCs display the characteristics of interstitial tissue DCs with respect to the expression of DC-associated molecules. The restricted presence of MR+MHCII+ cells in pLNs is suggestive of a peripheral tissue origin for the MR+ DCs and, because the traffic of DC into lymphoid tissues is known to increase after stimulation with microbes or their products (32–38), we considered the possibility that MR+ DCs are derived from MR+ cells present in the periphery (i.e., skin). To assess this possibility, we investigated the phenotype and behavior of MR+ cells in skin. Abundant MR+ cells were observed throughout the dermis of mouse ear skin, while cells expressing MHCII were restricted to the outer dermis and epidermis (Fig. 5A). Double immunofluorescence confirmed that dermal MR+ cells lack MHCII expression in situ, as no colocalization of MHCII and MR was observed (Fig. 5B). These results suggest that dermal MR+ cells are not phenotypically DCs in situ. Accordingly, when sections were double labeled for MR and CD68, a classical Mδ marker, the majority of MR+ cells coexpressed CD68 (Fig. 5B). Together, these data indicate that the MR is expressed by Mδ-like cells in the dermis of mouse skin. Explant studies were performed to determine whether dermal MR+ Mδs were capable of migration out of skin, an inherent property of DCs. Ears were mechanically split into dorsal and ventral sides, transferred into wells containing medium, and incubated at 37°C with 5% CO2 for 24 h. Migrated cells were collected and cytospins were prepared and labeled for MR and MHCII. We observed a notable heterogeneity in the expression of these two markers and found cells with high levels of MHCII and comparatively lower levels of MR (Fig. 6, top panels) as well as the opposite scenario (middle panels) and cells with intermediate levels of both markers (bottom panels). These differing phenotypes may represent cells at different stages of maturation. In some instances, MR+ cells displayed a dendritic morphology. These results indicate that MR+ Mδ-like cells can mobilize and acquire DC-like characteristics.

Targeting MR+ DCs in vivo
To investigate the function of the MR in MR+ DCs and the accessibility of these cells to Ag delivered in the periphery, we used purified rat anti-mouse MR mAbs as surrogate MR ligands to target MR+ cells in vivo (39). Preliminary targeting studies were conducted in naïve BALB/c mice where 15 μg of mAb (MR6F3, rat IgG2b anti-mouse MR) were injected s.c. in the upper forelimb close to the wrist area. The cervical, brachial, axillary, inguinal, popliteal and mesenteric LNs and the spleen were collected and processed for immunohistochemistry at various time points thereafter, from 30 min to 24 h postinjection. Injected mAbs were detected by incubating tissue sections with Alexa Fluor 488-labeled goat anti-rat IgG. These experiments indicated that the medullary cells in brachial, axillary and cervical LN were effectively targeted within 30 min postinjection. Conversely, targeting to paracortical MR+ DCs was poor and was only observed in the brachial LNs (the main draining LNs of this injection site), with only a few targeted cells being clearly visible at 24 h postinjection.

Additional experiments using anti-MR mAb, clone 6C3 (MR6C3, rat IgG2a anti-mouse MR), or IgG2a control IgG (clone GLIII/10) demonstrated that targeting to the paracortical region was dose dependent because no MR+ cells in T cell areas were targeted in any LN when 5 μg of mAb were used even though targeting to medullary cells still occurred (data not shown).

In view of the major effect that LPS had on the numbers of MR+ cells present in T cell areas, we injected BALB/c mice s.c. with 15 μg of purified anti-MR mAb, clone 6C3 (MR6C3, rat anti-mouse...
IgG2a), or IgG2a control IgG (clone GLIII/10) in the upper and lower forelimbs of mice treated i.v. with 5 μg of LPS or PBS 10 min earlier. We detected MR6C3 in the medullary regions of the cervical, brachial, axillary, and inguinal LNs of LPS- and PBS-treated mice. In PBS-treated animals few targeted cells were detected in the T cell areas of brachial or inguinal LNs (Fig. 7, A and B show representative inguinal LN). In the presence of LPS, the anti-MR mAb targeted numerous cells within the paracortical areas of brachial, axillary and inguinal LN (Fig. 7, C and D; a representative inguinal LN is shown). In all cases, targeted paracortical cells were MR⁺ and MHCII⁺, indicating specific delivery to MR⁺ DCs (Fig. 7, E–G). Delivery of anti-MR mAb was exclusively restricted to local draining LNs because no Ag could be detected in other nondraining lymphoid tissues such as, for example, the spleen (Fig. 7, H and I). No targeting of the rat IgG2a control Ab to LNs (shown in Fig. 7, A and C, insets) or spleen (Fig. 7, H and I, insets) was observed, indicating that no targeting system selective for rat IgG2a is present in secondary lymphoid organs. Similar results were obtained when the anti-MR mAb was injected s.c. in combination with LPS (1 μg/site), data not shown. Thus, MR⁺ DCs can acquire MR ligands delivered in the periphery with numbers of MR⁺ DCs in T cell areas containing MR ligands being increased in the presence of LPS.

**Generation of an anti-rat IgG Ab response after immunization with anti-MR mAbs**

Because we were able to target MR⁺ DCs specifically in vivo using rat anti-mouse MR mAbs, we sought to determine whether the delivery of Ag via the MR results in presentation to the adaptive immune system in an immunogenic fashion by assessing the generation of anti-rat IgG Abs in sera from s.c. immunized animals. Preliminary studies using 15 μg of MR6C3, MR5D3, and IgG2a indicated that no detectable response could be obtained in the absence of LPS and that 1 μg was better than 0.1 μg of LPS in promoting a humoral response (data not shown). These experiments also indicated that the mAb clone MR6C3 could elicit a more robust response than MR6C3.

To determine the optimal dose of mAb for the immunization studies, we immunized BALB/c mice s.c. in both forelimbs with varying doses of MR5D3 or control IgG2a in the presence of 1 μg of LPS. After 7 days the animals were sacrificed and serum was analyzed for the presence of anti-rat IgG by using ELISA. Animals immunized with MR5D3 consistently generated higher titers of anti-rat IgG than those immunized with the control protein. Significant differences in the anti-rat response were found between animals immunized with MR5D3 and control IgG2a at doses of 3.75 μg (p < 0.001) and 15 μg (p < 0.05). Based on these results, a dose of 3.75 μg of immunogen was chosen for use in future experiments (data not shown).

To confirm that the clone used for the immunization had an effect on the level of response obtained, animals were injected in both forelimbs with 3.75 μg of MR5D3, MR6C3, or isotype control in the presence of 1 μg of LPS s.c., and the presence of anti-rat IgG in the serum on day 7 was measured by ELISA (Fig. 8A).

**FIGURE 7.** Paracortical MR⁺ cells can be efficiently targeted in vivo using specific MR reagents in the presence of LPS. BALB/c animals were injected s.c. in the forelimb with rat anti-mouse MR6C3 or isotype control mAbs in the presence (C–G and I) or absence of LPS (A, B, and H). Secondary lymphoid tissues were collected 24 h later and processed for immunofluorescence. Injected mAbs were detected in tissue sections using a goat anti-rat IgG reagent (green). Tissue sections were also labeled for CD3 (red, A–E, H, and I), MR (red, F), or MHCII (red, G) to analyze the phenotype of targeted cells. T, T cell area; B, B cell follicle; rp, red pulp; wp, red pulp; DAPI, 4′,6′-diamidino-2-phenylindole.

**FIGURE 8.** Delivery of rat IgG to the MR using MR5D3 induces a more robust humoral response. A, Animals were immunized with either MR5D3 (▪), MR6C3 (▲), or control IgG2a (●) mAb in the presence of 1 μg of LPS. After 7 days, the serum was collected and the presence of an anti-rat IgG response was determined by ELISA. Immunization with MR5D3 generated significantly higher titers of anti-rat IgG compared with mice immunized with MR6C3 or IgG2a. Each symbol represents an individual animal. An asterisk (*) indicates significant differences.

B, The anti-rat response was predominately composed of IgG2a and IgG1 subclasses and immunization with MR5D3 induced a mixed Th1/Th2 response. Each symbol, the same as that used in A, represents an individual animal. An asterisk (*) indicates significant differences.
Differences between the responses induced by MR5D3 and IgG2a and by MR5D3 and MR6C3 were found to be highly significant ($p < 0.001$). These data indicate that Ag delivery through the MR, achieved by immunization with MR5D3 in the presence of LPS, induced improved Ab production compared with the control. Interestingly, the efficiency of anti-rat responses also appears to be dependent on the clone of mAb used.

Analysis of the presence of the IgG1 and IgG2a subclasses generated in immunized mice (Fig. 8B) showed that the animals immunized with MR5D3 generated stronger IgG2a and IgG1 responses compared with animals immunized with MR6C3 or the IgG2a control. The production of both IgG2a and IgG1 indicated that a mixed Th1/Th2 response was generated.

**Humoral responses to anti-MR mAbs are abrogated in MR$$^{-/-}$$ animals**

The specificity of B cell responses was confirmed by using MR$$^{-/-}$$ mice and wild-type (WT) C57BL/6 control animals. Animals were injected with 3.75 µg of either MR5D3 or control IgG2a in the presence (or absence in the case of WT animals) of 1 µg of LPS in both forelimbs, and sera were analyzed for total anti-rat IgG content by ELISA after 7 days (Fig. 9). In agreement with previous data, the induction of anti-rat IgG responses was dependent upon the presence of a microbial mimic. The results also show that the enhanced anti-rat IgG Ab production obtained in response to anti-MR mAb in WT animals (both BALB/c (Fig. 8) and C57BL/6 (Fig. 9)) was completely abrogated in MR$$^{-/-}$$ animals, indicating that these responses were MR-mediated and specific.

**Discussion**

In this study, we demonstrate that the delivery of soluble Ag in vivo through the MR leads to enhanced immunogenicity in the presence of innate stimulation. The mechanism behind this effect appears to involve the efficient uptake of MR ligands by a novel population of MR$$^{+}$$ DCs restricted to pLNs whose frequency is increased following treatment with TLR agonists. These cells are located in the paracortical areas of pLN and, based on phenotypical analysis by flow cytometry, correspond to a known DC subset that was thought to constitute dermal interstitial DCs (27). We propose that MR$$^{+}$$ DCs in pLNs are derived from MR$$^{+}$$ cells in the periphery. Results from the skin explant studies are consistent with this hypothesis, because a migratory population of MR$$^{+}$$ MHCI$$^{II}$$ cells with dendritic morphology is obtained even though the MR$$^{+}$$ cells located in the dermis have a M$$\delta$$-like phenotype (MHCI$$^{II}$$ CD68$$^{-}$$). No further stimulus apart from the physical dissociation of the dorsal and ventral sides of the ear was required to induce this migration. Presumably, increased migration would occur if an additional danger signal such as the presence of LPS or cytokines was also provided. Interestingly, tissues drained by mLNs also contain a large population of MR$$^{+}$$ M$$\delta$$s. MR$$^{+}$$ DCs in pLNs appear to be CD68$$^{-}$$ (data not shown), indicating that CD68 expression might be lost upon migration. The lack of MR-expressing cells in the paracortical region of mLNs indicates that lamina propria M$$\delta$$s do not migrate to draining mLNs or lose MR expression upon migration under the conditions tested.

Rat anti-mouse MR mAbs were used as specific surrogate ligands to probe the function of the MRs on DC in vivo. This approach has been previously used to deliver Ag via CD11c (40), MHCI$$^{II}$$ (41, 42), 33D1 (40), DEC-205 (43, 44), DC-SIGN (45), and FIRE and CIRE (46). Within 30 min postinjection, anti-MR mAbs could be detected in the medulla of LNs draining the site of injection, and by 24 h postinjection, MR$$^{+}$$ MHCI$$^{II}$$ cells containing anti-MR mAbs were detected within the paracortex.

The time lag that occurred before the targeted MR$$^{+}$$ DCs were detected in draining LNs suggests that these cells may have encountered anti-MR mAbs in the periphery before arrival into the LNs. Others have shown that the time taken for s.c. administered Ag to arrive in the LN is ~18 h, a similar time frame to that observed in our study (47). Moreover, unlike small molecules, the size of mAbs prevents their diffusion through the conduit system into the paracortical area (48), excluding the possibility of free mAbs draining directly into the paracortex and binding MR$$^{+}$$ DCs there. The targeting of MR ligands within the paracortex was dramatically improved if LPS was coadministered s.c. or if mice received LPS i.v. before the s.c. injection of mAb. These results are consistent with the increased egress of MR$$^{+}$$ cells from the dermis upon stimulation; these cells would internalize anti-MR mAbs in the periphery and transport them to the draining LNs. In contrast, MR$$^{+}$$ medullary cells were targeted in the presence or absence of LPS. It is likely that free anti-MR mAbs drained from the site of injection via the lymph into the subcapsular sinus of the local LN before entering the conduit system to the medulla. The targeting properties of the anti-MR mAb in vivo differ from those of anti-DEC-205 Ab, which diffuses throughout the secondary lymphoid tissues after s.c. injection (49). Ag delivery to MR$$^{+}$$ DCs was exquisitely restricted to lymphoid tissue draining the site of injection and did not extend to either the spleen, the mLNs, or the pLN located on the contralateral side of the animal, even in the presence of LPS. This is likely to be due to Ab clearance by MR$$^{+}$$ medullary cells, which would remove the majority of the injected mAbs reaching the LN in free form. Clearance of anti-MR mAbs by medullary cells would not only prevent Ag access to other lymphoid tissue but, more importantly, to other APCs. In this way this removal system would limit the acquisition and presentation of MR ligands to the adaptive immune system. These results are consistent with previous studies describing the lack of MR expression in DC in vivo under steady-state conditions (8) and a major defect in homeostatic clearance (3, 50) but normal immunity against Candida albicans or Pneumocystis carinii, both MR ligands, in MR-deficient mice (50, 51).

**FIGURE 9.** Enhanced anti-rat IgG responses obtained against anti-MR mAb are abrogated in MR$$^{-/-}$$ animals. MR deficient mice or C57BL/6 WT control mice were immunized with MR5D3 (■) or control IgG2a (▲) mAb in the presence of LPS. Groups of C57BL/6 were also immunized with MR5D3 or control IgG2a alone. Serum was collected after 7 days and the presence of the anti-rat IgG was determined by ELISA. No anti-rat response was detected in mice receiving mAb alone, whereas significant responses were found in those animals that received MR5D3 and LPS compared with control protein. The enhanced anti-rat IgG response obtained in response to MR5D3 was abrogated in MR$$^{-/-}$$ mice, as MR5D3 and control IgG2a elicited similar responses in these animals. Each symbol represents an individual animal. An asterisk (*) indicates significant differences.
After identifying the conditions under which MR could be exploited by professional APCs to internalize Ag for presentation to the adaptive immune system in vivo, anti-MR mAb were used to determine whether the enhanced targeting of MR ligands to DCs in T cell areas correlated with the generation of enhanced humoral responses. This strategy involved the immunization of animals with purified rat IgG preparations and analysis of anti-rat IgG responses in the serum of injected animals.

In the presence of LPS, a single s.c. dose of rat anti-mouse MR mAb induced significant anti-rat IgG production compared with the isotype control mAb. The enhanced anti-rat response was completely abrogated in MR−/− mice, illustrating the capacity of MR+ cells to promote B cell responses and the specificity of the system. These results provide the first conclusive in vivo evidence of a role for MR in the induction of adaptive immune responses. Interestingly, differential humoral responses were induced by immunization with the MR5D3 and MR6C3 clones. Both of these mAbs recognize the C-type lectin-like carbohydrate recognition domain 4-7 region of the MR, but it is not known whether the binding affinities of these clones differ and/or whether their intracellular handling is different. This requires further investigation but may reflect results in the human system where differential engagement of the MR on moDCs by anti-human MR mAbs induces differential programs of activation. This was also shown to occur for some natural ligands (52).

It will also be important to determine the exact contribution of MR+ DCs in the generation of humoral responses, given that Mφs bearing ligands for the CR domain have been shown to bind injected CR domain-bearing fusion proteins in the subcapsular sinus, potentially mimicking the delivery of Ags by soluble MRs (53). In addition to direct targeting to MR+ cells in the draining LNs, it is possible that the mAbs injected in this study also bound to free soluble MR and were delivered to CR domain-ligand+ cells located in the subcapsular sinus. In the presence of stimulation, targeted CR domain-ligand+ cells would migrate into B cell follicles (28, 30) and present native Ags in complex with soluble MR to differentiating B cells in the germinal center. However, the pattern of anti-MR mAb targeting in vivo is not consistent with the delivery of mAbs to CR domain-ligand+ cells via soluble MR. Furthermore, the soluble MR Ag delivery pathway would not be favored under the conditions tested here because CR domain multimerization is required for optimal targeting to CR domain-ligand+ cells in vivo (53) and, given that the anti-MR mAb is probably in a monomeric form, it would be an unsuitable ligand for inducing CR domain multimerization.

The induction of CD4+ or CD8+ T cell responses via Ag delivery through the MR was not addressed in this study and is the focus of future work. However, indirect evidence from the data presented here suggests that T cells can become activated and assist in the process of Ig isotype switching in response to MR ligands. During the course of these studies a role for MR in the cross-presentation of soluble OVA in vitro and in vivo has been suggested by others (19). Intriguingly, in this work the authors demonstrate a defect in the uptake by CD11c+ cells and the cross-presentation of soluble OVA in the spleen and bone marrow of MR-deficient animals. These results are not in agreement with our studies demonstrating the lack of MR expression in splenic DC by both immunofluorescence and flow cytometric analysis of splenic CD11c+ cells (data not shown). Future studies using chimeric anti-MR mAbs bearing CD4 and CD8 epitopes will clarify this issue, because the contribution of other putative receptors or a defect in DC function in the absence of MR can be ruled out by using this system.

In this study, we demonstrate the presence of a previously unknown murine MR+ DC subpopulation whose numbers are controlled by innate stimulation. These cells are most likely derived from myeloid skin leukocytes that can mobilize and acquire a DC phenotype under appropriate stimulation. Efficient targeting of MR ligands to MR+ DC takes place when LPS is present, and this correlates with an enhanced induction of humoral responses against these ligands. These data provide the first in vivo evidence of a role for MR in Ag presentation to the acquired immune system and reveal potential pathways available for endogenous molecules recognized by the MR to be presented in an immunogenic form to the acquired immune system. Moreover, the correlation between the immunogenicity of MR ligands and the presence of surrogate signs of infection (e.g., endotoxin) observed in our studies parallels the triggering effect that infection can have in the induction of autoimmune diseases and thus place the MR in a pivotal position in the induction of autoimmunity.

The benefits obtained from exploiting a homeostatic receptor as an Ag acquisition system by immunogenic DC would be derived from its usefulness in increasing the sampling ability of APCs. Because the MR has a well established ability to bind pathogen-derived products and, when expressed on DCs, is able to target Ag for presentation (see Ref. 54 for review), the existence of a highly regulated and restricted MR-mediated Ag presentation pathway in the context of infection would ensure the recognition of microbial products that could otherwise escape presentation due to efficient clearance. In this way, the presentation of endogenous molecules would be minimized and this, together with effective induction of central and peripheral tolerance, will limit the generation of pathological immune responses.

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References


