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Antigenic Targeting of the Human Mannose Receptor Induces Tumor Immunity[1]

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Pattern recognition receptors are preferentially expressed on APCs allowing selective uptake of pathogens for the initiation of antimicrobial immunity. In particular, C-type lectin receptors, including the mannose receptor (MR), facilitate APC-mediated adsorptive endocytosis of microbial glyconjugates. We have investigated the potential of antigenic targeting to the MR as a means to induce Ag-specific humoral and cellular immunity. hMR transgenic (hMR Tg) mice were generated to allow specific targeting with the anti-hMR Ab, B11. We show that hMR targeting induced both humoral and cellular antigenic specific immunity. Immunization of hMR Tg mice with B11 mAbs induced potent humoral responses independent of adjuvant. Injection of hMR Tg mice with mouse anti-hMR Ab clone 19.2 elicited anti-Id-specific humoral immunity while non-Tg mice were unresponsive. B11-OVA fusion proteins (B11-OVA) were efficiently presented to OVA-specific CD4 and CD8 T cells in MR Tg, but not in non-Tg, mice. Effector differentiation of responding T cells in MR Tg mice was significantly enhanced with concomitant immunization with the TLR agonist, CpG. Administration of both CpG and B11-OVA to hMR Tg mice induced OVA-specific tumor immunity while WT mice remained unprotected. These studies support the clinical development of immunotherapeutic approaches in cancer using pattern recognition receptor targeting systems for the selective delivery of tumor Ags to APCs. *The Journal of Immunology,* **2007, 178: 6259–6267.**

The mannose receptor (MR[4]; CD206) is a member of the calcium-dependent lectin receptor (CLR) family, which has characteristic carbohydrate recognition domains with selective binding to specific glycans (1, 2). Due to the properties in its carbohydrate pattern recognition, its potent capacity of endocytosis, and its role in phagocytosis of microorganisms, MR has been suggested to play a dual role in host defense and homeostasis (3–5). MR selectively recognizes polysaccharides terminated in mannose, fucose, or *N*-acetylglucosamine that are abundant on microbes and some endogenous glycoproteins and subsequently contributes to their internalization and clearance. The role of MR in homeostasis of serum glycoproteins has been conclusively demonstrated through the development of MR-deficient mice (6). However, the importance of MR in the innate and adaptive immune responses to pathogens is less clear. Some studies support an important role for MR in this capacity (7, 8), while other investigations suggest MR is not critical for protection against selected microbial pathogens (9, 10). Adding to the complexity, a number of recent studies suggest that MR cross-linking elicits anti-inflammatory responses, and thus may down-regulate innate inflammatory (11–13).

MR is expressed primarily by tissue macrophages (Mϕ) and lymphatic and hepatic endothelia in humans and mice (1, 2). MR is also expressed by subsets of dendritic cells (DC), primarily interstitial DCs, as well as on cultured DCs from human monocytes and mouse BM (14, 15). The expression of MR on APCs has led to the suggestion that it may be involved in Ag processing and presentation. The MR property to recycle constitutively allows a rapid and successive accumulation of its natural ligands or mannosylated Ag into intracellular compartments that may then access the processing pathways for MHC presentation. Indeed, a number of studies support a role for MR in generating both MHC class II- and MHC class I-restricted immune responses (16). These investigations have used mannose or mannan conjugated to various forms of Ag to promote Ag uptake by MR. In vitro studies have shown that MR targeting can enhance the efficiency of MHC class II presentation to Ag-specific T cells by several orders of magnitude, relative to nontargeted Ag (17, 18). In addition, conjugation of the human tumor Ag (mucin 1) to oxidized-mannan induced Th1 and CTL responses and complete tumor protection in mice (19, 20). However, the overlapping pattern recognition of mannose-type ligands between MR and other CLRs, such as DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), makes the interpretation of the MR contribution to the observed immune responses difficult (3, 21).

In addition to ligands, Abs that recognize APC surface molecules have been used as vehicles to deliver Ags in efforts to enhance immune responses and trigger cross-presentation (22). Rapid, potent, and adjuvant-independent humoral responses have

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[4] Abbreviations used in this paper: MR, mannose receptor; CLR, calcium-dependent lectin receptor; Mϕ, macrophage; DC, dendritic cell; hMR, human MR; BAC, bacterial artificial chromosome; muMR, mouse MR; BM, bone marrow; PEC, peritoneal exudate cell; DT, diphtheria toxin; WT, wild type.

been successfully elicited by the direct targeting of Ags coupled to Abs specific for FcRs, class II MHC, and CD11c (23–26). Expansion and activation of Ag-specific $CD4^+$ and $CD8^+$ T cells with the same approaches or with immune complexes were also achieved in T cell culture systems and some animal studies (27–32). In particular, Ab targeting of Ag to DEC-205, a CLR abundantly expressed by DC, resulted in profound peripheral T cell tolerance or immunity, depending on the maturation/activation status of the DC (33–35).

We have previously described the development of a human mAb (B11) specific for the human MR (hMR), and fusion proteins consisting of this Ab and different tumor-associated Ags for targeted Ag delivery (36, 37). As described for mannosylated Ags, Ab targeting of Ags to MR resulted in significant enhancement of MHC class II presentation to Ag-specific T cells than nontargeted Ag. Moreover, human DCs that were primed with B11-targeted fusion proteins elicited MHC class I-restricted CTL activity against tumor cell lines expressing the relevant Ag. These studies, which used either the melanocyte differentiation Ag pmel17 or the oncofetal Ag human chorionic gonadotropin β-subunit, unambiguously confirmed that MR targeting combined with appropriate activating signals can lead to enhanced Ag processing and presentation to CD4 and CD8 T cells in vitro.

The present study was undertaken to extend these observations to an animal model. Therefore, we have engineered mice to express the hMR for the investigation of B11 and other anti-hMR Abs. These mice provide the additional advantage of comparison to nonexpressing littermates, which permits the unambiguous interpretation of the MR contribution to any observed effect. We have successfully introduced the entire gene for hMR, and explored the effects of Ag delivery in vivo. In this study, we report the characterization of transgenic mice expressing hMR (hMR Tg) and the induction of tumor protective immune responses as a result of in vivo antigenic targeting to MR.

Materials and Methods

Generation of hMR Tg mice

The hMR Tg mice were made in the C57BL/6 strain using standard microinjection techniques. Based on genomic sequence information on the 30-exon *hMR* gene (38), basic local alignment sequence tool sequence searches were conducted on National Center for Biotechnology Information (NCBI) and several human bacterial artificial chromosome (BAC) clones corresponding to this region were identified. One in particular, 211-kb BAC clone RP11-457D2, containing the 5′ and 3′ extremes of the exon sequence previously identified by Kim et al. (38), was obtained from Research Genetics, and characterized further. Sequence verification of exons 1 and 30 were conducted; in addition, sequence identity of a region −26 kb upstream of exon 1 was verified. Full-length *Not*I-digested BAC insert DNA was prepared as per Chrast et al. (39). This DNA was used for microinjection into fertilized C57BL/6 oocytes to generate hMR Tg mice.

Routine PCR genotyping of mouse tail genomic DNA prepared by the Roche Magnapure method (Roche Diagnostics) used PCR Supermix (Invitrogen Life Technologies) with primers located in the 5′ and 3′ end of the BAC insert DNA (732bp amplicon from −26 kb upstream of exon 1 and 547 bp corresponding to exon 30). The hMR Tg mice were expanded and housed under pathogen-free conditions. WT mice were littermates of Tg or age- and gender-matched C57BL/6 mice purchased from Taconic Farms. CD11c-DTR ((Itgax-DTR/GFP)57Lan/J) mice (C57BL/6 background) were obtained from The Jackson Laboratory and crossed with hMR Tg mice.

MR Abs

The B11 mAb (a human IgG1) were purified from supernatants of transfected Chinese hamster ovary cells as previously described (36, 37). B11-OVA fusion proteins were made as an in-frame fusion of the entire OVA cDNA at the C terminus of the B11 Ig H chain. Mouse anti-hMR Ab clones 19.2 and 15-2 were purchased from BD Biosciences/BD Pharmingen and Serotec, respectively. Anti-mouse MR (muMR) MR5D3 was obtained from BD Biosciences/BD Pharmingen. All reagents/Abs that were used in vivo or in cultures were endotoxin-free (<10 EU/mg, tested by *Limulus* amebocyte lysate assay), either purchased as functional grade or run through the endotoxin removal gel column (Pierce Biotechnology).

Preparation of peritoneal and bone marrow (BM)-derived cells

Peritoneal exudates cells (PEC) were elicited by i.p. injection with 1 ml of 3% thioglycolate 4 days before harvesting. BMDCs cells were generated from BM harvested from femurs and tibias flushed with RPMI 1640 medium. After depletion of the lineage-positive cells, BM precursor-enriched cells were cultured with 10% FBS, 50 μM 2-ME, 10 ng/ml GM-CSF in RPMI 1640 medium at 3×10^6 cells/well in 6-well plate. The cultures were fed at days 2 and 4 by gently swirling the plates, aspirating 75% of medium and adding back fresh complete medium containing GM-CSF (PeproTech). On day 6, nonadherent cells were removed by gently swirling plates and aspirating cultured medium. Adherent cells were harvested by incubation with Versene (Invitrogen Life Technologies). The majority of these cells were positive for the expression of CD11c as determined by flow cytometry (data not shown). hMR expression was evaluated flow cytometrically with anti-hMR mAbs clone 19.2 and by Western blot analysis with anti-hMR mAb clone15-2.

In vivo Ag targeting and immunohistochemical analysis

Mice were injected s.c. in the two front paws with 5 μg of B11 mAb in 5 μl of PBS to each side. The same amount of irrelevant human IgG1 (Sigma-Aldrich) was administered s.c. as a control for FcR interactions. The skin near and distal to the injection site, proximal (axillary) and distal (inguinal) lymph nodes, spleen, liver, and lung were harvested 24 h later. These organs were snap-frozen and sectioned for immunohistochemical staining with Abs against human IgG. To assess the expression profiles of hMR in the Tg mice, cryostat sections from a panel of 14 different tissues were stained with B11 or 19.2 following the methods published previously (36).

Immunization for humoral responses

For humoral responses, mice of 8–12 wk of age were immunized i.p. with 0.1or 10 μg of B11 in 100 μl of PBS or in 100 μl of Ribi adjuvant per mouse at 2-wk intervals, for a total of three doses. The anti-hMR 19.2 mAb (mouse IgG1) in combination with 100 μl of Ribi was administered to evaluate the generation of anti-Id Abs. Each group consisted of a minimum of five hMR Tg mice or age and gender-matched wild-type (WT) mice. Blood specimens were collected from orbital sinus under anesthetics on day 0 for baseline and 1 wk after each injection and sera were isolated and stored at −20°C for ELISA.

ELISA

Anti-B11 titers were measured by standard ELISA using immobilized B11 on microtiter plates and AP-conjugated goat anti-mouse IgG Fcγ Ab (Jackson ImmunoResearch Laboratories) for detection. Plasma specimens were diluted serially and titers were determined by the highest dilution of each specimen giving an absorbance at OD_{405} that was greater than double the value of control mouse serum. Ab subclass determination was done by means of ELISA with SBA clonotyping system/AP (Southern Biotechnology Associates). For anti-Id responses microtiter plates were coated with 19.2 $F(ab)_2$ or irrelevant isotype-matched $F(ab)_2$ and blocked with 5% BSA. Anti-Id IgG specific for 19.2 were detected by a goat anti-mouse IgG, Fc-specific probe. Pooled plasmas from immunized mice were tested for blocking of 19.2 mAb binding to hMR by flow cytometry. Human monocyte-derived DC were incubated with 19.2-PE alone or in the presence of the plasma pool at various ratio at 4°C for 1 h.

In vivo T cell proliferation assays

$CD45.1^+$ OT-I and OT-II cells were labeled with CFSE (Molecular Probes) and 5×10^6 cells were injected i.v. into recipient mice before immunization. On day 3 after transfer flow cytometric analysis identified OT-I and OT-II populations among splenocytes using the following mAbs (BD Pharmingen): anti-CD45.1 PE (A20) and either anti-CD4-PerCP-Cy5.5 (GK1.5) or anti-CD8α-PerCP-Cy5.5 (53-6-7). The percentage of divided OT-I or OT-II T cells was calculated as the $CFSE^+$, $CD45.1^+$, $CD4^+$ or 8^+ population outside the unimmunized single peak/total $CD45.1^+$, $CD4^+$ or 8^+ population. For systemic DC depletion, RIP-mOVA/CD11c-DTR mice were treated i.p. with 4 ng/g body weight of diphtheria toxin (DT; Sigma-Aldrich) 18 h before transfer of T cells. One dose of DT leads to 48–72 h of $CD11c^+$ cell depletion (40).

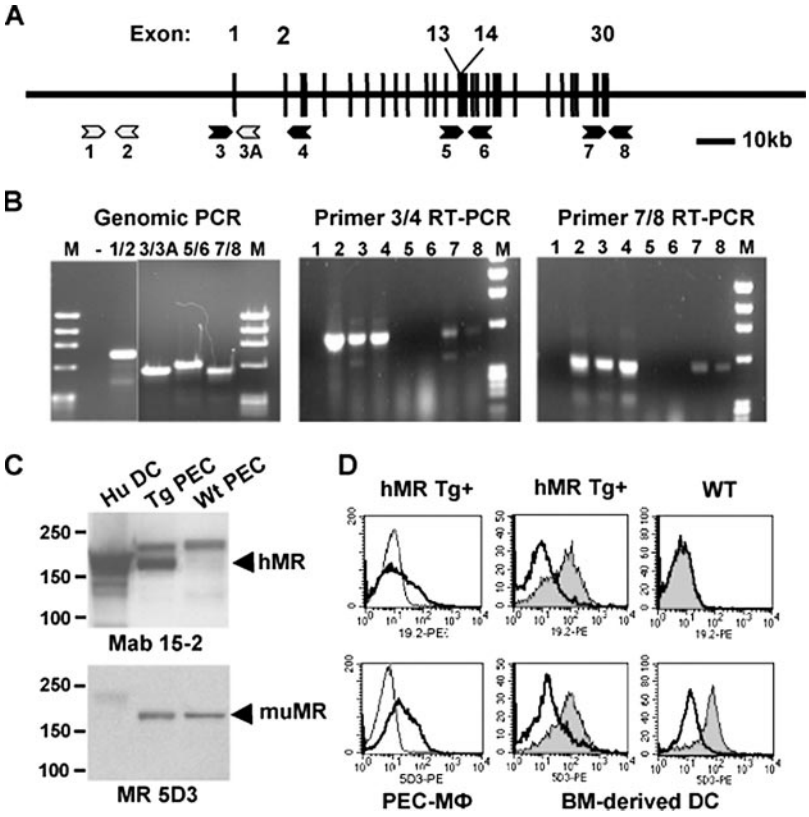

FIGURE 1. hMR transgene and its expression in mice. *A*, Schematic map of the 211-kb BAC clone corresponding to the hMR. Exons are indicated by vertical lines. The arrowheads beneath the map indicate positions of PCR primers used for sequence confirmation and PCR genotyping. Black arrowheads are specific to exon sequences; gray arrowheads correspond to intronic or nontranscribed regions. *B*, PCR genotyping and RT-PCR amplification of mRNA. *Left panel*, Primer combinations as defined in Fig. 1*A* were used on genomic tail DNA from F_1 mouse 44 and are indicated over the lanes. M, phiX 174 + *Hae*III markers; (−), no input primers. *Middle* and *right panels*: 1, no input RNA; 2, human placental poly(A)$^+$ RNA; 3, F_1 mouse 44 BM total RNA; 4, F_1 mouse 44 spleen total RNA; 5 and 6, non-Tg spleen total RNA; 7, F_1 mouse 43 BM total RNA; 8, F_1 mouse 43 spleen total RNA; M, phiX 174 + *Hae*III markers. *C*, Western blot analysis with Abs specific for hMR (15-2) or muMR (MR5D3) on cell lysates from PEC-Mϕ derived from hMR Tg and WT mice, and human DCs. Precision plus protein standards were included as molecular mass markers. The band corresponding to hMR and muMR are indicated with arrowheads. *D*, Cell surface expression of MR. Cells from hMR Tg and WT mice were stained with hMR-specific (19.2-PE) or muMR-specific (MR5D3-PE) Abs (bold line for PEC-Mϕ and shaded histograms for BM-derived DC). Mouse IgG1-PE and rat IgG2a-PE were used as isotype controls. PEC-Mϕ were the gated population that were negative in lineage markers Ter-119, Gr-1, CD3ε, and B220, while BM-derived DC were gated on all viable cells.

IFN-γ intracellular staining

Effector responses were assessed 5 days after transfer of CD45.1$^+$ OT-I cells in mice immunized with B11-OVA with or without CpG (InvivoGen). A total of 2×10^6 splenocytes/500 μl of complete medium were incubated with 0.1 μM SIINFEKL peptide and GolgiStop solution (BD Biosciences) for 4 h at 37°C. Cell fixation and permeabilization was done using the Cytofix/Cytoperm Plus kit (BD Biosciences) before intracellular staining with anti-IFN-γ-Alexa Fluor-647 (XMG1.2). Dot plots show the percentage of IFN-γ cells among CD45.1$^+$, CD8$^+$ gated cells.

ELISPOT

Mice were immunized on days 0 and 14 with 10 μg of B11-OVA with or without 50 μg of CpG (InvivoGen) both given i.p. Splenocytes were harvested on day 21 and plated at 3×10^5 cells/well on a Millipore 96-well membrane plate coated overnight with anti-IFN-γ capture Ab (AN18) and blocked with 10% FBS in RPMI 1640 (Mabtech). Cells were incubated overnight with either no Ag or OVA (300 μg/ml). Wells were washed and incubated with biotinylated anti-IFN-γ (R4-6A2-biotin) and streptavidin-alkaline phosphatase and developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate. A CTL Immunospot Series 3A Analyzer was used for imaging and counting with Immunospot software.

Tumor prevention model

Mice were immunized on days 0 and 14 with 10 μg of B11-OVA with or without 50 μg of CpG both given i.p. On day 21 mice were injected s.c. with 2×10^5 M04 tumor cells (57). Mice were monitored for tumors until sacrifice at day 18 and three-dimensional tumor sizes measured with calipers. A Student *t* test was used to evaluate significance of growth inhibition of immunization vs no treatment.

Results

Generation of hMR Tg mice

To establish an animal model enabling antigenic targeting of the hMR in vivo hMR Tg mice were generated. A 211-kb BAC clone containing the entire *hMR* gene and its 5′ and 3′ flanking regions was integrated into the genome of C57BL/6 mice (Fig. 1*A*), inclusive of 56 kb of 5′ flanking regulatory sequences upstream of exon 1, allowing for transgene expression under the control of its own promoter. Multiple PCR primer pairs established that the transgene was integrated intact and real-time PCR analysis was consistent with a single copy insertion (data not shown) in the founder line. The expression of the transgene was confirmed using RT-PCR analysis and hMR-specific Abs in several immunoassays (Fig. 1*B*). Immunoblotting analysis on lysates derived from activated PEC revealed a ~180 kb band only in the hMR Tg mice, which corresponded to the size of hMR in lysates from human DC (Fig. 1*C*). The endogenous muMR, detected with species specific reagents, was expressed at similar levels in hMR Tg mice and WT mice in PEC-Mϕ and BM-derived DC, indicating that bearing the transgene did not interrupt the endogenous MR expression (Fig. 1*D*). Immunostaining with the human anti-hMR mAb B11 in a panel of 14 tissues, including skin, lymph node, spleen, thymus, liver, lung, brain, heart, kidney, colon, ileum, ovary, oviduct, and testis, demonstrated a distribution pattern of hMR (Fig. 2*A* and data not shown) consistent with that previously described in human tissues (5, 15) and with the reported expression of muMR (5, 41, 42). These cells were distributed in capsular, trabecular, and interstitial connective tissues, as well as around vessels. In particular, the specific immunoreactivity with mAb B11 was observed in sinusoidal endothelium in the spleen and liver, as well as tissue-specific Mϕ, such as alveolar Mϕ in the lung, and Kupffer cells in the liver. Immunofluorescence studies demonstrated colocalization of B11 in MHC class II$^+$ APCs of the skin which likely represent dermal Mϕ and DCs (Fig. 2*B*).

APC targeting with the anti-hMR mAb B11

To accomplish specific targeting of the hMR in vivo the anti-hMR mAb, B11 was injected s.c. into WT and hMR Tg mice. In the draining lymph node of Tg mice, hMR-targeted cells were mainly localized to medullary sinusoids and cords, and particularly in a

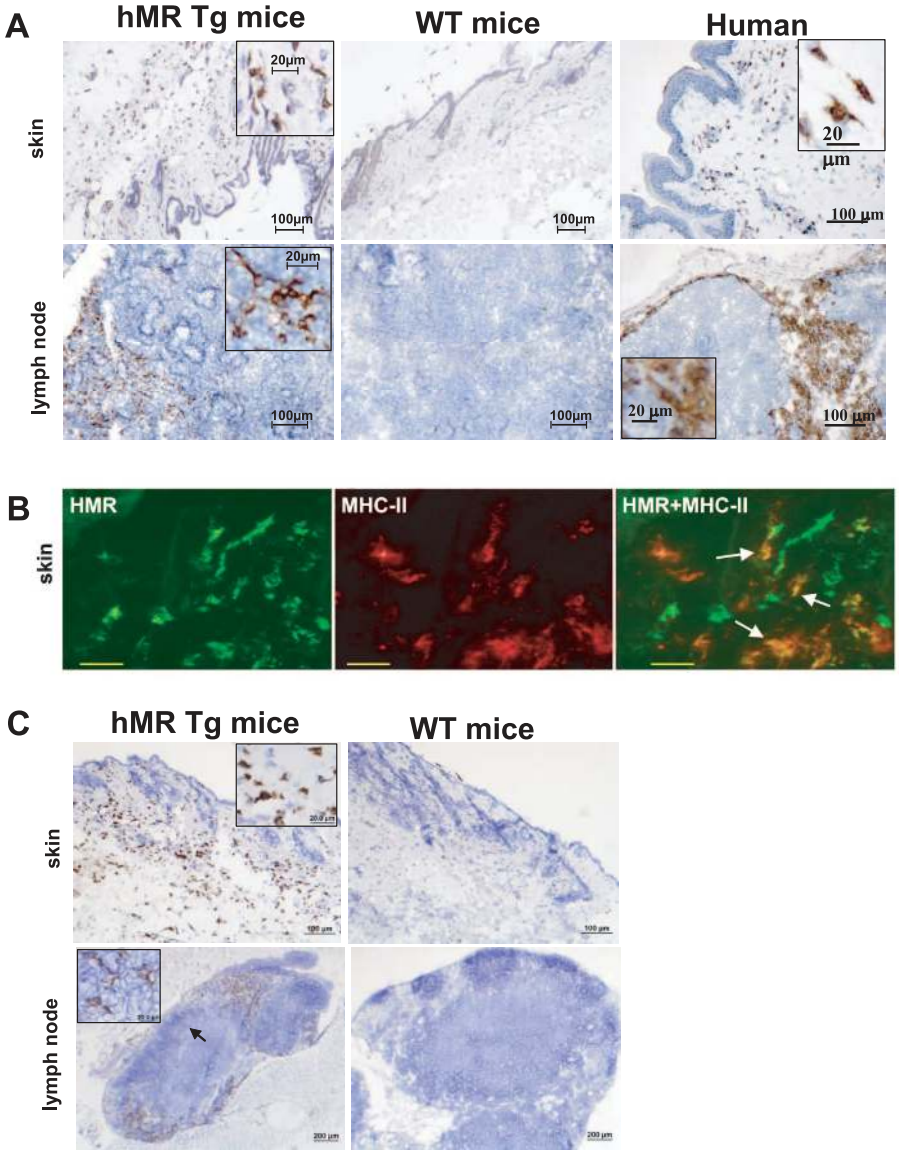

FIGURE 2. In vivo targeting of hMR. *A*, Immunohistochemical localization of hMR expression in humans and hMR Tg and WT mice. Comparable expression patterns in hMR Tg mice, but not WT mice, are seen in human skin and lymph node. *B*, hMR is expressed by MHC class II^+ cells in the skin. Acetone-fixed cryostat sections were stained with FITC-conjugated anti-hMR and biotinylated anti-MHC class II mAb, and subsequently incubated with Alexa 488 goat anti-FITC Ab or Cy3-strepavidin. *C*, Draining lymph node and the skin of injection site were harvested from hMR Tg mice 24 h after intradermal administration of anti-hMR mAb B11 (5 μg). Staining with rabbit anti-human IgG demonstrates prominent B11 uptake in the epidermis and in the lymph node, specifically the capsular sinus and in medullary structures.

subset of scattered clusters of cells in paracortical regions (Fig. 2*C*). In the splenic red pulp, hMR targeted cells were readily apparent in hMR Tg mice but not in WT mice (data not shown).

Induction of humoral responses to hMR-targeted Ag

hMR Tg mice and their WT littermates were immunized with B11 to determine the effect of MR targeting on humoral immunity (Fig. 3*A*). Administration of 10 μg of B11 i.p. with a single immunization, even in the absence of adjuvant, was sufficient to generate high-titered anti-B11 IgG responses in hMR Tg mice that were further inducible with boosting with second and third rounds of immunizations. Immunological memory in hMR Tg mice was apparent since high titered responses were induced upon recurrent immunization, some 3 mo after the final boost. Under the same stimulations, anti-B11 human responses were undetectable in WT mice demonstrating hMR targeting.

Enhancement of humoral responses was also investigated using the murine anti-hMR mAb 19.2. The Id of murine mAb can serve as a model of a poorly immunogenic Ag in mice, as the induction of anti-Id immune responses generally requires conjugation to an immunogenic carrier protein such as keyhole limpet hemocyanin or genetic fusion with GM-CSF (43, 44).

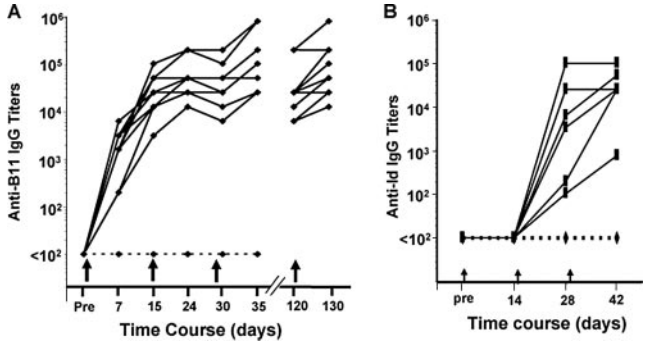

FIGURE 3. Antigenic targeting to MR enhances humoral responses. *A*, B11 mAb 10 μg without adjuvant was administered i.p. on days indicated by arrows. Serum anti-B11 titers were measured by ELISA at the indicated time points. Solid and dashed lines represent individual hMR Tg and WT mice (10 mice/group), respectively. *B*, Induction of anti-Id responses in hMR Tg mice. A total of 10 μg of mAb 19.2 mixed with 100 μl of Ribi was administered i.p. on days indicated by arrows. The anti-19.2 $F(ab')_2$ titers were analyzed by ELISA using a murine IgG-Fc-specific secondary Ab. Solid and dashed lines represent individual hMR Tg and WT mice (six mice per group), respectively.

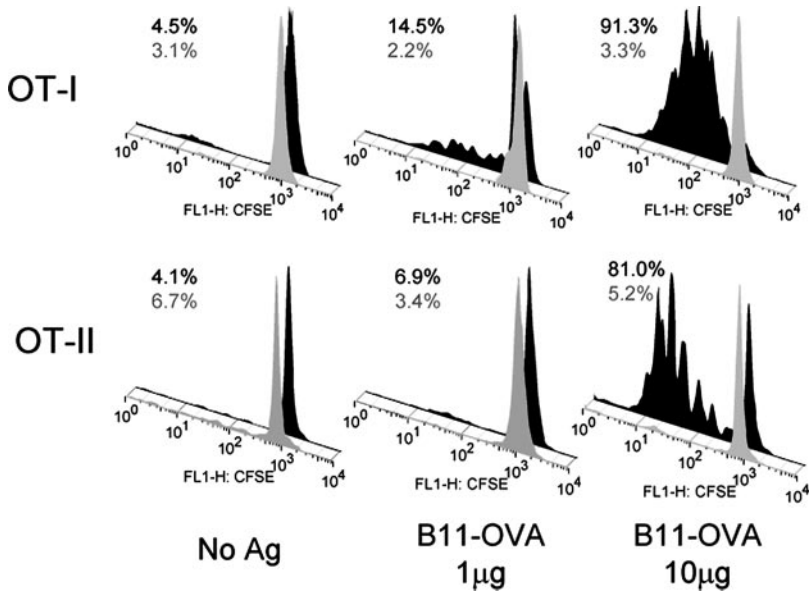

FIGURE 4. hMR-targeted Ags access the exogenous and cross-presentation pathway in vivo. WT (gray) or hMR Tg (black) mice were injected with either CFSE-labeled OT-I or OT-II T cells and subsequently injected i.p. with PBS, 1 or 10 μg of B11-OVA. Splenocytes were obtained 72 h later for flow cytometric analysis. Percentages of divided OT-I or II cells are shown. Data representative of three mice/group are shown. Immunization with B11-OVA-induced OT-I and OT-II brisk proliferative responses occurred in hMR Tg mice and were absent in WT mice.

Humoral responses to 19.2 mAb administered with Ribi adjuvant were readily generated in hMR-Tg but not WT mice (Fig. 3*B*). The anti-19.2 IgG titers were predominantly Id-specific, based on specificity for 19.2 and the ability to block 19.2 binding to human monocyte-derived DC (data not shown). Importantly, the anti-Id titers were boosted by subsequent immunizations, despite the presence of potentially neutralizing Abs. Taken together, these data confirm that targeting to hMR with a human or a murine mAb can enhance humoral immunity to the targeted Ag, presumably by enhancing the efficiency of Ag uptake, processing, and presentation.

hMR-targeted Ag accesses the exogenous and cross-presentation pathway in vivo

To directly assess the ability of hMR targeting to promote T cell priming in vivo, a B11-OVA fusion protein was genetically engineered containing an in-frame fusion of the entire OVA cDNA at the C terminus of the B11 Ig H chain. This produced a fusion protein of the predicted 232 kDa, containing two OVA molecules per B11 IgG1 molecule. Generation of the B11-OVA fusion molecule enabled the use of immunization models using naive Tg OT-I and II cells, two well-characterized Tg TCR systems that, respectively, allow evaluation in vivo of MHC class I- and II-restricted OVA-specific T cell priming.

C57BL.6 recipients of either CFSE-labeled naive TCR Tg OT-I CD8 or OT-II CD4 cells were immunized i.p. with either 1 or 10 μg of B11-OVA. In WT mice, OT-I cells failed to proliferate with either dose of B11-OVA (Fig. 4). In contrast, B11-OVA immunization induced robust and dose-dependent OT-I proliferative responses in hMR Tg mice; 14 and 91% of OT-I cells had divided in hMR Tg mice injected with either 1 or 10 μg of B11-OVA, respectively. CD4 OT-II proliferative responses were not detected with 1 μg of B11-OVA in either WT or hMR Tg mice but when mice were immunized with 10 μg of B11-OVA, OT-II proliferative responses were strongly induced in hMR Tg mice (81% divided) but not in WT mice. Thus, hMR targeting accesses both the exogenous and cross-presentation pathway in vivo allowing priming of both MHC class I- and MHC class II-restricted T cell responses.

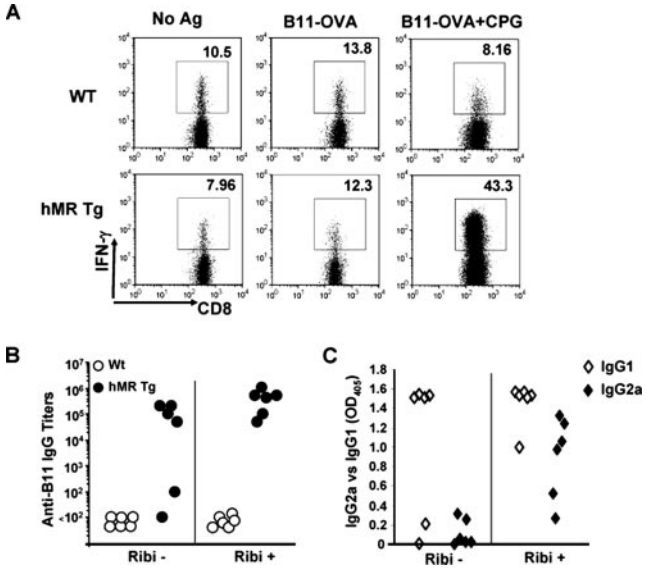

FIGURE 5. Adjuvants enhance hMR-targeted immune responses. *A*, CpG enhances OT-I effector differentiation. OT-I recipients were immunized i.p. with 10 μg of B11-OVA with or without 50 μg of CpG. Splenocytes were harvested 5 days later, restimulated for 4 h with OT-I (257–264) peptide, and stained intracellularly for IFN-γ production. Data are expressed as the percent of IFN-γ^+ producers among CD45.1$^+$, CD8$^+$ gated input cells. *B*, Ribi adjuvant enhances Ab responses upon low dose of targeting Ag. B11 mAb (0.1 μg) with or without Ribi was administered i.p. at 2-wk intervals in hMR Tg and WT mice. Anti-B11 titers were measured by a murine total IgG-specific ELISA on sera harvested 1 wk after the third immunization. Each dot denotes an individual mouse and the experiment is representative of three separate studies. *C*, Ribi adjuvant promotes Th1-type humoral responses. The identical sera of immunized mice were analyzed by ELISA using IgG1 and IgG2a subclass-specific secondaries. Although anti-B11 responses of the IgG1 subclass were produced upon B11 immunization in the presence or absence of Ribi adjuvant, IgG2a responses were only induced in Ribi recipients.

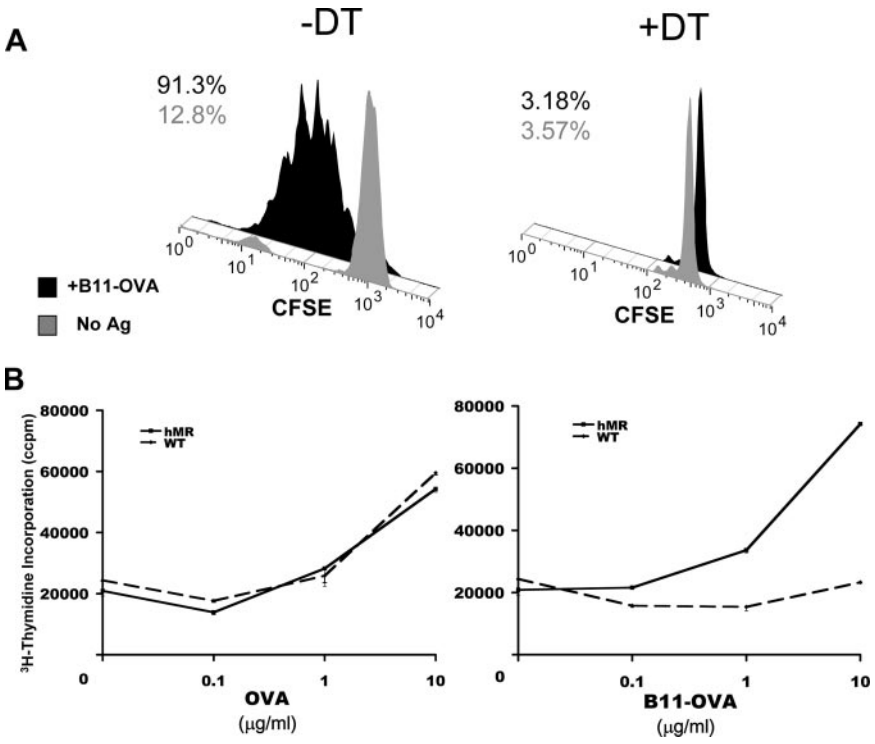

FIGURE 6. hMR-targeted cross-presentation requires DCs in vivo. *A*, Flow cytometric analysis of splenocytes isolated from DT-treated or untreated hMR Tg/CD11c-DTR mice 3 days posttransfer of CFSE-labeled OT-I cells alone or together with 10 μg of B11-OVA (*right panels*). Histograms are gated on $CD8^+CFSE^+$ cells and represent one of three independent experiments. OT-I cell proliferative responses were abolished in the absence of DCs. *B*, hMR Tg BMDCs mediate cross-presentation by B11-OVA. BMDCs cultured from BM precursors for 7 days were treated with 10 μg/ml CpG and cultured with OT-I CD8 Tg T cells (DC:T ratio of 1:20) and graded doses of soluble OVA or B11-OVA mAb. Thymidine incorporation was measured after 36 h. In the absence of CpG, hMR Tg BMDCs failed to induce detectable B11-OVA-mediated OT-I proliferation (data not shown).

hMR targeting induces adjuvant-dependent effector T cell priming

Targeting of APCs in vivo using another Ag uptake receptor, DEC-205, resulted in T cell proliferation without concomitant effector differentiation (34). To assess whether OT-I cell proliferative responses were accompanied by effector CD8 differentiation in hMR-targeted mice, WT or hMR Tg mice OT-I recipients were immunized i.p. with 10 μg of B11-OVA either in the presence or absence of the TLR 9 ligand CpG as adjuvant. After 5 days, splenocytes were restimulated with OT-I peptide, and IFN-γ production evaluated by intracellular staining to assess OT-I effector differentiation (Fig. 5*A*). In WT mice, there was no evidence of increased OT-I effector differentiation after immunization with B11-OVA in either the presence or absence of CpG. In contrast, there was robust induction of IFN-γ production by OT-I cells in hMR Tg mice immunized with B11-OVA in the presence of CpG. Notably, immunization of hMR Tg mice with B11-OVA alone failed to induce OT-I IFN-γ production. Thus, despite the robust proliferative response seen after immunization of hMR Tg mice with B11-OVA alone (Fig. 4) there was no concomitant enhancement of IFN-γ effector differentiation, which instead required addition of the TLR 9 agonist CpG.

To evaluate whether the addition of a strong adjuvant could enhance the hMR-targeted humoral response, WT and hMR Tg mice were immunized once with a low dose of B11 (0.1 μg). In WT mice no detectable humoral responses were detectable even in the presence of a strong adjuvant (Fig. 5*B*). Robust humoral responses occurred in hMR Tg mice with IgG1 being the predominant isotype in the absence of adjuvant. However, in the presence of adjuvant (Ribi) humoral responses were quantitatively and qualitatively enhanced (Fig. 5*C*). Importantly, induction of significant IgG2a titers required immunization together with adjuvant. Thus, hMR targeting induces strong humoral responses, however, Th1-type isotype switched responses requires addition of adjuvant.

T cell priming in vivo by hMR targeting requires DCs

DCs have been implicated as the relevant cross-presenting cell for effector CD8 T cell priming. Multiple potential APCs express MR in vivo including DC. To determine the contribution of DCs to hMR-targeted priming, hMR Tg mice were mated with CD11c-DT receptor (DTR) Tg mice that express a human high-affinity DTR on the surface of $CD11c^+$ cells, making them specifically sensitive to DT-mediated apoptosis (40). Using this DC depletion model, DCs have been previously shown to be required for the cross-presentation of cell-associated (40) and soluble exogenous Ag (45), as well as for the presentation of bacterial (40) and viral Ags (46, 47). Preliminary experiments in DT-treated WT and CD11c-DTR mice, showed that transferred OT-I cells accumulated and localized to the T cell zones of the lymph nodes and spleen in comparable numbers regardless of the presence or absence of $CD11c^+$ cells (data not shown). Therefore, DT-treated hMR Tg mice and hMR Tg/CD11c-DTR mice were transferred with 5×10^6 CFSE-labeled OT-I cells, injected with B11-OVA and proliferation was assessed after 3 days. Strikingly, no proliferation of the OT-I cells was seen in DC-depleted recipient mice (Fig. 6). Although roles for subpopulations of marginal zone splenic macrophages cannot be ruled out (48), these data are consistent with a major contributing role for DCs in hMR-mediated T cell priming in vivo. We confirmed the ability of hMR targeted Ag to mediate MHC class I-restricted Ag presentation by BMDCs in vitro, although the induction of OT-I proliferative responses in vitro by B11-OVA Ab was not seen without the addition of CpG (data not shown).

hMR-targeted Ag can induce protective tumor immunity

The capacity of B11-OVA vaccines to target hMR-dependent Ag-specific CD8 responses was further evaluated in tumor immunity models. WT and hMR Tg mice were immunized twice with 10 μg

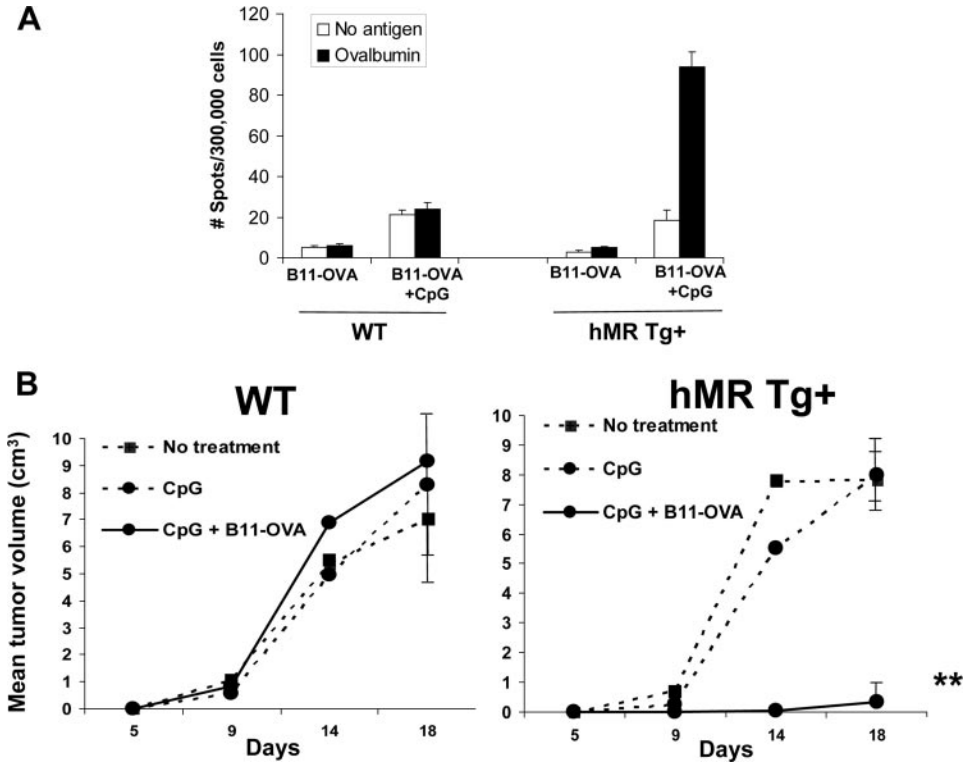

FIGURE 7. hMR-targeted vaccines induce tumor immunity. *A*, IFN-γ effector responses after B11-OVA immunization requires addition of adjuvant. Mice were immunized twice with 10 μg of B11-OVA, 50 μg of CpG, or both on days −21 and −7; splenocytes were harvested on day 0. Splenocytes were stimulated with either no Ag or 300 μg/ml OVA overnight and IFN-γ production was assessed by ELISPOT. Data are expressed as the mean of three wells. *B*, Mice were immunized as above and challenged s.c. with B16-OVA tumor cells. Mean tumor sizes of five mice per group are shown. Tumors in mice immunized with B11-OVA in combination with CpG were significantly smaller on day 18 ($p = 0.009$, Student's t test (one-sided)).

of B11-OVA in the presence or absence of CpG. One week following the secondary immunization, OVA-specific T cell responses were evaluated with IFN-γ ELISPOTs. Consistent with the results seen using the OT-I system, IFN-γ responses only occurred in hMR Tg mice immunized with both B11-OVA and CpG (Fig. 7*A*). Humoral responses of the IgG1 subclass were induced in all hMR Tg mice immunized with B11-OVA, however, as was the case with Ribi adjuvant (Fig. 5), IgG2a responses required concomitant immunization with CpG (data not shown). Thus, B11-OVA and CpG immunization induced cellular effector responses and this immunization protocol was further evaluated in a tumor prevention model. Mice were immunized twice with B11-OVA and CpG and challenged s.c. with 2×10^5 MO-4 cells, a B16F10 melanoma OVA transfectant. In WT mice, tumors grew rapidly in all mice regardless of immunization strategy. However, in hMR Tg mice significant tumor growth inhibition was exhibited in mice immunized with B11-OVA and CpG adjuvant ($p = 0.009$ vs no treatment) (Fig. 7*B*). Thus, immunization strategies incorporating both adjuvant and hMR targeting of tumor Ag can induce protective tumor immunity.

Discussion

We developed Tg mice expressing the entire *hMR* gene under the control of its own promoter to functionally assess the immunological consequences of hMR antigenic targeting. In these mice, the hMR expression profile, including the distribution pattern and regulation, mimics the reported expression pattern for MR in mice and humans. The expression of the endogenous muMR was similar in hMR Tg and WT mice. Additionally consistent coexpression of hMR and muMR using dual staining on tissue sections or isolated cells from hMR Tg mice, further confirmed the appropriate distribution of the transgene. Injected anti-hMR Abs were readily detectable in lymphoid and nonlymphoid tissues of hMR Tg mice but not in non-Tg localizing primarily to subcapsular regions in draining lymph nodes.

Specific delivery and loading of Ag in hMR Tg was achieved using the anti-hMR human IgG1 Ab, B11 which is currently under clinical investigation as a vaccination approach in cancer (16, 22, 36). This effort is supported by our results which establish proof-of-principle in mice that hMR targeting in vivo can accomplish cross-priming of Ag-specific responses sufficient to induce protective tumor immunity. These in vivo data derived from mice studies extend our previous observations that targeting Ags to MR on human DCs in vitro allows for efficient presentation to human CD4 and CD8 T cells (36, 37).

Our results demonstrate that hMR targeting induce both humoral and cellular immunity in hMR Tg mice. Regarding the humoral response, immunization with either low-dose B11 or with a murine anti-hMR mAb expected to have reduced immunogenicity was further enhanced with Ribi, a strong adjuvant. These results are consistent with previous studies demonstrating enhanced humoral immunity with Ab-targeted vaccines directed to other receptors on APC, including MHC molecules, FcRs, and integrins (24, 25, 49).

We demonstrate with OT-I and OT-II Tg T cell systems and the B11-OVA fusion protein that hMR receptor targeting accesses the exogenous and cross-presentation pathway in vivo. Immunization of hMR Tg mice with B11-OVA-induced robust OT-I and OT-II proliferative response whereas no proliferative responses occurred in non-Tg WT animals indicating that other endocytic pathways were not efficiently targeted at the doses of B11-OVA used here, including Fc and complement receptors as well as the endogenous muMR which was recently identified genetically (50), as an uptake receptor for OVA in vivo. Notably, in these studies of Burgdorf et al. (50), effective Ag presentation of OVA via the muMR required 30-fold higher amounts of OVA than that used here in our studies (100–150 μg of soluble OVA vs 1–10 μg of B11-OVA containing 0.37–3.7 μg of OVA). Thus, both humoral and CD4 and CD8 cellular responses can be induced with Ab-mediated antigenic targeting to hMR, indicating that Ags acquired through

hMR targeted can be presented in vivo to both B and T lymphocytes and accesses both the exogenous and cross-presentation pathways.

However, although both Ag-specific proliferative T cell responses and IgG1 humoral responses were driven efficiently by hMR targeting, concomitant effector differentiation and Th1-type humoral responses required coadministration of an adjuvant. Indeed, in the absence of adjuvant, the robust T cell expansion seen at day 3 was no longer evident on day 5 (data not shown). Although this could potentially be explained by emigration of effectors to peripheral tissues, the contraction following initial T cell activation in the absence of adjuvant is more likely due to deletion of responding T cells.

These data are similar to that seen after immunization of mice with conjugate proteins targeting another MR family member, DEC-205. As shown here, both effector OT-I responses and effective tumor immunity in the M04 prevention model was accomplished by anti-DEC-205 OVA immunization only in the presence of anti-CD40 mAb agonists (35) consistent with the notion that tumor vaccines require concomitant antigenic targeting to APCs and an APC activation signal.

The MR and related CLRs have distinct distribution patterns that include specific APC populations important in controlling and regulating immune responses. Targeting Ags to these endocytic receptors on APC represents an attractive strategy to enhance the efficacy of vaccines, as they are able to guide exogenous protein Ags into vesicles that efficiently process the Ag for MHC class I and class II presentation. Although abundant on cultured DC, MR is not found on many of the classical APC populations of mice in situ such as splenic DC and Langerhans cells (41, 51). Furthermore, MR^+ cells are generally not associated with the T cell areas of lymphoid organs (52). Therefore, it is unclear which APC are involved in enhancing immune responses in hMR-targeted mice. Our findings showing that OT-I proliferative responses in vivo were abolished by in vivo elimination of DCs in CD11c-DTR Tg mice are consistent with a primary role for DC in hMR-mediated cross-priming, although a role for subpopulations of marginal zone splenic macrophages cannot be ruled out (48). It appears that under naive conditions, only a subset of the MR^+ cells that capture Ag are likely to be involved in the direct presentation of the MR-targeted Ag. Linehan (53) reported the MR-positive interstitial APC in multiple nonlymphoid organs coexpressed MHC class II, and the colocalization of these two molecules in the same subcellular compartments within a proportion of the APC. In agreement with previous observation of MR and MHC class II dual-positive cells scattered in the paracortex in lymph nodes (41), we detected cells that internalized hMR-targeted Ag were MHC class II positive in the same regions of lymph nodes. Use of agents that effect MR expression and activation of APC may significantly alter the distribution pattern, migrations, and Ag-presenting capacity of MR-expressing cells. Indeed, CpG was required for effective cross-presentation by hMR-targeted BMDCs in vitro.

The shared ligands between some CLR have made interpretation of the MR contribution to immune responses difficult to assess. Our use of hMR-specific Abs avoids potential involvement of other CLRs. Because MR-expressing APC must express Fc and complement receptors, it is likely that the immunological outcome of MR-targeted Ab vaccines containing an intact human IgG1 Fc domain will not be exclusively regulated only by hMR-mediated uptake but will also be influenced by coengaged Fc and/or complement receptors (54–56).

Taken together, this study provides in vivo evidence that targeting Ag to MR can lead to enhanced humoral and cellular responses sufficient to induce tumor immunity. Understanding the specific APC involved in this process and how to manipulate their activity with activating agents will be important to optimize vaccination strategies for cancer or other diseases.

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Disclosures

Several authors are current or past employees of either Mederex or Celldex (a subsidiary of Mederex). The animal model evaluated in this study provides mechanistic insight into potential anticancer biological pathways used by B11-based and other Ab-based therapeutics currently under clinical investigation at Celldex.

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