

MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells

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Like CD1d-restricted *i*NKT cells, mucosal-associated invariant T cells (MAITs) are “innate” T cells that express a canonical TCR α chain, have a memory phenotype, and rapidly secrete cytokines upon TCR ligation. Unlike *i*NKT cells, MAIT cells require the class Ib molecule MHC-related protein I (MR1), B cells, and gut flora for development and/or expansion, and they preferentially reside in the gut lamina propria. Evidence strongly suggests that MAIT cell activation is ligand-dependent, but the nature of MR1 ligand is unknown. In this study, we define a mechanism of endogenous antigen presentation by MR1 to MAIT cells. MAIT cell activation was dependent neither on a proteasome-processed ligand nor on the chaperoning by the MHC class I peptide loading complex. However, MAIT cell activation was enhanced by overexpression of MHC class II chaperones li and DM and was strikingly diminished by silencing endogenous li. Furthermore, inhibiting the acidification of the endocytic compartments reduced MR1 surface expression and ablated MAIT cell activation. The importance of the late endosome for MR1 antigen presentation was further corroborated by the localization of MR1 molecules in the multivesicular endosomes. These findings demonstrate that MR1 traffics through endocytic compartments, thereby allowing MAIT cells to sample both endocytosed and endogenous antigens.

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Abbreviations used: BFA, brefeldin A; CMA, concanamycin A; CRT, calreticulin; MAIT, mucosal-associated invariant T cell; MFI, mean fluorescence intensity; MIIC, MHC class II compartment; MR1, MHC-related protein I; sh, small hairpin; si, small interference.

Mucosal-associated invariant T cells (MAITs) are a unique “innate” T cell population that expresses an invariant mV α 19 TCR α in mice and the homologous hV α 7.2 in humans (1). With the exception of preferential localization in the intestine, MAIT cells bear striking similarities to the extensively studied *i*NKT cells that express mV α 14 in mice and hV α 24 in humans (2, 3). Functional analysis of MAIT cells has been difficult because of the paucity of this population in mice. However, studies using recently derived V α 19 i TCR transgenic mice have shown that MAIT cells have a memory or activated phenotype (CD44^{hi}, CD45RB^{lo}, CD25^{lo}, and CD62L^{neg}) and are ICOS^{int-hi} (4). Like *i*NKT cells, MAIT cells expand poorly in vitro upon TCR ligation, but rapidly release IFN- γ , IL-4, -5, and -10 (4, 5). Although the function of MAIT cells has yet to be fully elucidated, it has been speculated that they serve as regulatory

cells in a feedback loop controlling gut IgA production (2, 3). Additionally, MAIT cells have been implicated in the regulation of autoimmune inflammation in a mouse EAE model (5). More specifically, adoptive transfer of transgenic MAIT cells inhibited the induction and progression of EAE and was accompanied by reduced production of inflammatory cytokines and increased production of IL-10. Interestingly, the increased IL-10 was accounted for, at least in part, by an ICOS-mediated interaction between MAIT cells and B cells. Thus, accumulating evidence suggests that MAIT cells are potent immune regulatory cells that can shift the balance between homeostasis and autoimmune responses.

Whereas NKT cell development is CD1d dependent, MAIT cell development and/or expansion is dependent on MHC-related protein I (MR1), B cells, and gut commensal flora. In addition, we have shown that select MAIT cell hybridomas are activated by cells

The online version of this article contains supplemental material.

overexpressing transfected MR1 protein in a manner that can be blocked by either mAb to the TCR or MR1 (2, 6). In these *in vitro* assays, several lines of evidence suggested that MR1 presents a ligand to MAIT cells (6). Intriguingly, the $\alpha 1$ and $\alpha 2$ domains of the MR1 molecule are highly conserved among mammals, with $\sim 90\%$ identical residues between human and murine MR1 proteins (7). This degree of homology is much higher than $\sim 60\text{--}70\%$ for the CD1 and class Ia molecules, and strongly suggests that MR1 presents an evolutionarily conserved ligand (8). However, the chemical nature of the MR1 ligand remains unknown. Interestingly, recent reports suggest that the synthetic glycolipid α -mannosyl ceramide and its derivatives can differentially activate V $\alpha 19i$ T cells (9, 10). However, comparisons between MR1 and class Ia molecules indicate that the putative ligand-binding groove of MR1 is more homologous to classical class I molecules and contains more hydrophilic or charged residues than do CD1 molecules (8). Thus, it remains critical to define the physiological ligands presented by MR1.

Because pathogens can reside in disparate intracellular compartments, the immune system has evolved distinct antigen presentation pathways for sampling various intracellular organelles. MHC class Ia molecules are typically loaded within the ER with peptides derived from proteasome-digested proteins and transported into the ER lumen by TAP. Within the ER lumen, peptide loading by class I is facilitated by physical association with TAP, as well as other members of the peptide-loading complex, e.g., tapasin, calreticulin (CRT), and ERp57 (11). In contrast, nascent MHC class II molecules physically associate with the invariant chain (Ii), which prevents ligand binding in the ER and promotes trafficking to a late endosomal compartment called the MHC class II compartment (MIIC) (12, 13). Within the MIIC, the class II molecule associates with DM, which facilitates the release of an Ii-associated peptide and binding of an endosomal peptide (12, 14).

Extending the MHC paradigms, CD1 isoforms have evolved elaborate presentation pathways to sample the lipid content of disparate organelles, elucidated in part with CD1-overexpressing cells (15). Specifically, CD1 molecules bind a self-lipid in the ER, transit to the cell surface, and then recycle to bind lipid in an endocytic compartment. Interestingly, for the CD1d isoform, physical association with Ii plays a redundant role with sorting motifs in the CD1d tail, either of which promotes endosomal trafficking to bind appropriate lipids (16). Distinct lipids appear to be loaded onto different CD1 isoforms depending on the specific endosomal compartment in which loading occurs; as with MHC molecules, chaperones and enzymes are critical factors (15). Interestingly, a high percentage of *i*NKT hybridomas are autoreactive, detecting either an unknown self-ligand or the recently identified self-lysosomal glycolipid iGb3 (17–19). Furthermore, presentation of self-CD1d ligands has been speculated to play a central part in the NKT cell development, infections, and autoimmunity (19, 20).

Like *i*NKT cells, select MAIT cell hybridomas can be activated by transfected cells expressing high levels of MR1 with

presentation of the self-ligand (2, 6). In this study, we used these hybridomas to demonstrate that MAIT cell activation by MR1 does not require proteasome function, TAP, tapasin, or CRT. However, MR1 physically associates with Ii and DM, and this association promotes MR1 localization in endosomal compartments. More strikingly, we present multiple lines of evidence showing that both MR1 trafficking through late endocytic compartments and the presence of Ii are critical for MAIT cell activation, but have less impact on MR1 surface expression. Thus, MR1 appears to use an antigen presentation pathway similar to MHC class II and/or CD1, potentially allowing it to sample endocytic compartments containing endogenous and microbial antigens.

RESULTS

MR1 surface expression and MAIT cell activation are TAP independent

MR1 message (7) and protein (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20072579/DC1>) are ubiquitously expressed in various cell lines and tissues. However, we and others have thus far failed to identify cells expressing endogenous MR1 on the cell surface using multiple anti-MR1 antibodies or that are capable of MAIT cell activation (21) and (unpublished data). These combined findings suggest that, unlike the constitutively expressed CD1 molecules

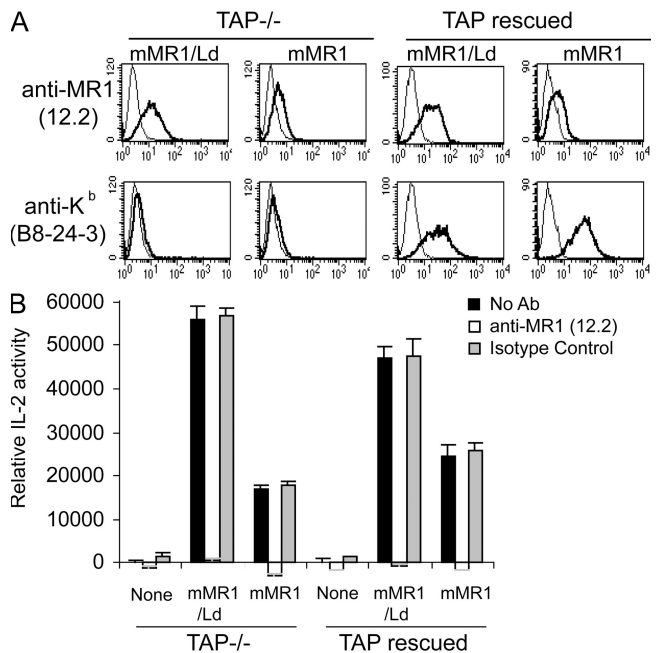


Figure 1. MR1 surface expression and MAIT cell activation are not dependent on TAP expression. The TAP-deficient cell TAP^{-/-} and the TAP-rescued cell were transduced with wild-type mMR1 or the mMR1/L^d chimeric molecule. (A) Thin lines indicate the background with secondary antibody only; thick lines indicate the staining with annotated antibodies. (B) Activation of MAIT cell hybridoma 6C2 (mean \pm the SD) was observed with no additional antibody (No Ab), anti-MR1 blockade, or isotype-matched irrelevant Ab (34–2-12), as applied in other MAIT cell activation assays. Data are representative of two experiments.

(15, 17), MR1 requires an unknown regulatory mechanism for surface expression. However, select MAIT cell hybridomas are activated by various transfectants overexpressing MR1 (unpublished data), thus providing a model system for studying how an endogenous antigen is presented to MAIT cells. As noted, such a model system has interesting parallels with CD1d presentation of self-ligands to *i*NKT cells (17).

Using this model system, we first addressed the role of TAP for MR1 expression and MAIT cell activation. Interestingly, MR1 molecules were previously detected in association with TAP and the peptide-loading complex at levels similar to classical class Ia molecules (22). However, MAIT cells were found to be more abundant in TAP-deficient mice (1, 2). To test the role of TAP in MAIT cell activation, TAP1-deficient cells were transduced with retrovirus containing a cDNA-expressing intact mMR1 molecules or an mMR1/L^d chimera. This chimera has the $\alpha 1/\alpha 2$ domains of mouse MR1 and the $\alpha 3$ domain of L^d molecule (22). Its inclusion was based on the fact that ligand binding should be unaltered, yet its surface expression is higher than intact mMR1, possibly because of better interaction with $\beta 2m$ (22). As shown in Fig. 1 A, the absence or presence of TAP did not affect the expression of either intact or chimeric mMR1 molecules. As a control, expression of H-2K^b was shown to be TAP dependent. In spite of their disparate levels of expression, neither intact mMR1 nor the MR1/L^d chimera was dependent on TAP for MAIT cell activation (Fig. 1 B). Importantly, in these functional assays, MAIT cell activation was shown to be MR1 specific using control cells not transduced with MR1 and a mAb specific for folded MR1 to block

MAIT cell activation. Thus, MR1 surface expression and MAIT cell activation are clearly TAP independent.

MR1 surface expression and MAIT cell activation do not require tapasin, CRT, or class Ia molecules

Ligand binding of MHC class Ia is facilitated by its association with the peptide-loading complex consisting of TAP, tapasin, CRT, and ERp57 proteins (11, 23). Of particular note, both tapasin and CRT can associate with class Ia in the absence of TAP, and both of these ER chaperones promote assembly of class I heavy chains with peptide and $\beta 2m$ (11). Therefore, it was of interest to examine whether these ER chaperones might also affect MR1 expression and MAIT cell activation. Cell lines deficient for tapasin or CRT were transduced with intact or chimeric MR1 and further tested for MR1 surface expression and MAIT cell activation. Results showed that neither tapasin (Fig. 2, A and B) nor CRT (Fig. 2, C and D) affected MR1 expression or MAIT cell activation. To determine whether class Ia molecules influence MR1 expression and MAIT cell activation, intact or chimeric MR1 was introduced into fibroblasts that were genetically deficient for K^b and D^b expression (KODO). As shown in Fig. 2 (E and F), class Ia-deficient or wild-type cells expressed similar levels of intact and chimeric MR1 and stimulated MAIT cells equally well. This finding indicates that MR1 is unlike the class Ib molecules Qa-1 and HLA-E that bind and present leader peptides of class Ia molecules. Furthermore, this finding also suggests that the low level of surface MR1 expression does not result from an unfavorable competition with class Ia molecules for ER chaperones or ligands.

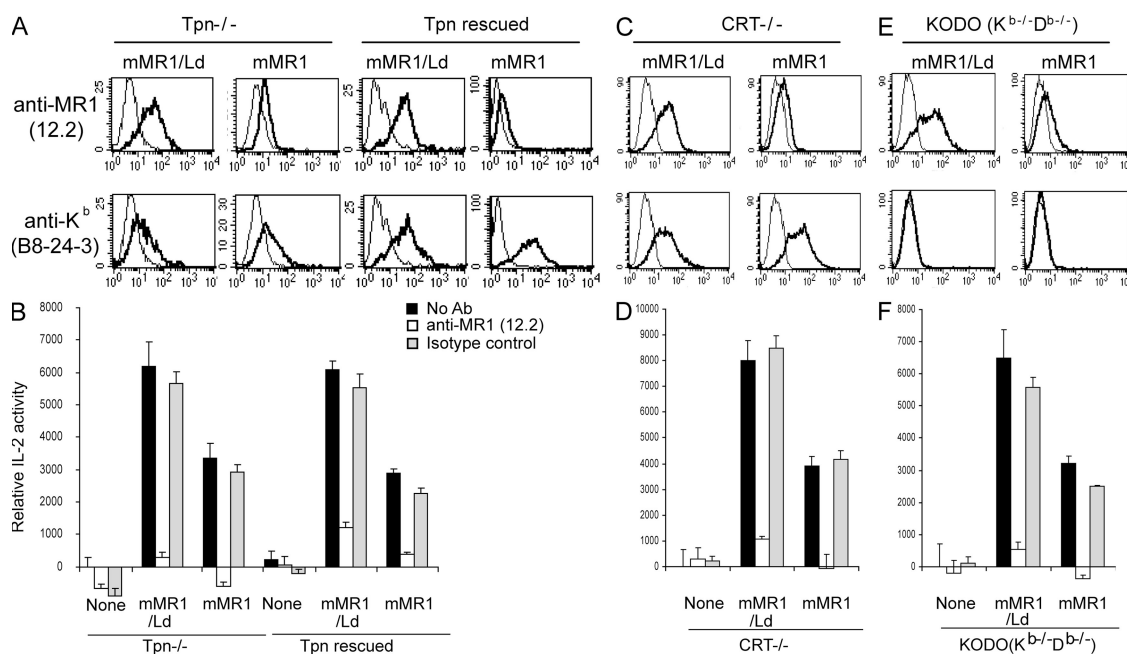


Figure 2. MR1 surface expression and MAIT cell activation are not dependent on tapasin, CRT, or class Ia molecules. The tapasin-deficient cell (Tpn^{-/-}), tapasin-rescued cell (Tpn^{-/-}Tpn), CRT-deficient cell CRT^{-/-} (K42), and K^b D^b knockout cells (KODO) were transduced with mMR1 or the mMR1/L^d chimera and tested for MR1 surface expression (A, C, and E) or MAIT cell activation (B, D, and F; shown as the mean \pm the SD).

MR1 surface expression and MAIT cell activation is proteasome independent

The ubiquitin–proteasome pathway is the principle mechanism by which cytoplasmic proteins are turned over and peptide ligands are generated for binding to MHC class Ia molecules. Accordingly, inhibition of proteasome function blocks peptide generation and antigen presentation by MHC class Ia molecules to CD8⁺ T cells (24). Although TAP independence makes it less likely that MR1 binds a proteasome-cleaved peptide, it does not formally rule it out because there are examples of class Ia presentation of TAP-independent, but proteasome-dependent, peptides (25). To determine whether MR1 expression and MAIT cell activation are proteasome-dependent, MR1-transduced LM1.8 and WT3 cells were treated with lactacystin or NLVS (NIP-L₃VS) (26). Neither of these proteasome inhibitors had an impact on MR1 expression (Fig. 3 A) or MAIT cell activation (Fig. 3 B), although both inhibitors reduced expression of class Ia molecules K^b and L^d (Fig. 3 A) and inhibited activation of the T cell hybridoma L3, which is alloreactive for the L^d molecule (Fig. 3 B). In these comparisons, we also included the drug brefeldin A (BFA), which blocks protein transport past the mid-Golgi complex (27). Overnight treatment with BFA had similar effects on MR1 and class Ia L^d or K^b cell surface expression, suggesting that they have similar rates of surface turnover (Fig. 3 A). As expected, activation of both MR1- and L^d-reactive hybridomas was ablated after overnight BFA treatment, indicating that surface expression of and consequent T cell activation by both MR1 and class Ia molecules requires protein transport from the ER to the Golgi complex. However, these assays demonstrate that, unlike class Ia mole-

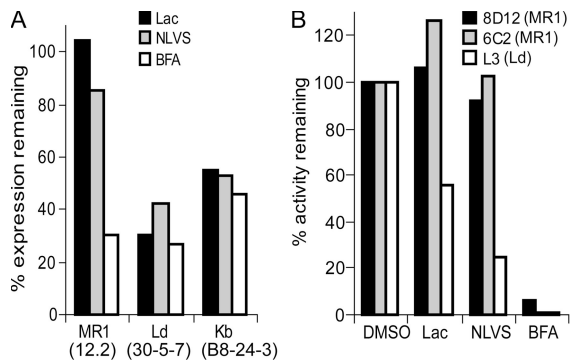


Figure 3. Proteasome function is not required for MR1 surface expression or MAIT cell activation. The mMR1-, L^d-, or K^b-expressing LM1.8 cells were treated with the specific proteasome inhibitors Lactacystin (Lac; 25 μM) and NLVS (50 μM) and BFA (10 μg/ml), using DMSO as a vehicle control. (A) Surface molecules were stained with indicated antibodies. The percentage of the remaining MHC molecules on the surface was calculated by dividing mean fluorescence intensity (MFI) of the inhibitor-treated cells with that of the control. (B) MAIT cell hybridomas 6C2 and 8D12 responding to inhibitor-treated cells were compared with L^d-reactive hybridoma L3. T cell activity was shown as the percentage to that of the control. Data shown are representative of two experiments.

cules, MR1 expression and MAIT cell activation are proteasome independent.

Ii and DM are associated with MR1 and enhance MAIT cell activation

Because class I chaperones were not required for activation of MAIT cells by MR1, we asked whether class II chaperones provide assistance. To determine whether MR1 expression

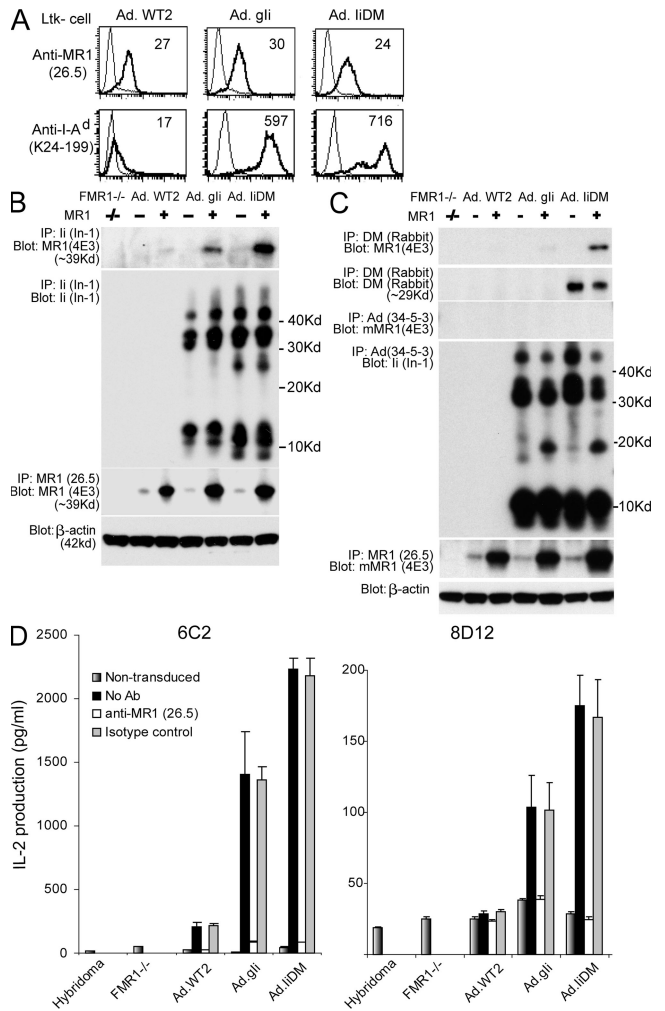


Figure 4. Ii and DM are associated with MR1 and facilitate MAIT cell activation. Ltk⁻ cells overexpressing of A^d, Ii, and DM (13) were transduced with mMR1. (A) Thick lines indicate the staining with annotated antibodies; thin lines indicate the negative control with secondary antibody only. Numbers indicate MFI of antibody staining. These data are representative of multiple experiments. (B) Coprecipitation of Ii with MR1 with indicated antibodies using the MR1-deficient fibroblast FMR1^{-/-} as a negative control and β-actin staining as a loading control. These experiments were repeated two times with similar results. (C) Coprecipitation of MR1 with DM, but not with MHC class II molecule I-A^d. (D) Activation of MAIT hybridomas 6C2 and 8D12 mean ± the SD) with cells overexpressing Ii was quantified using a standard curve generated with recombinant mouse IL-2. Nontransduced indicates cells without MR1 transduction; No Ab indicates no additional antibody. Eight experiments were done, with similar results.

was affected by overexpression of Ii and DM, intact MR1 was introduced into Ltk⁻ cells expressing class II and Ii (A^d.gli), or class II, Ii, and DM (A^d.IiDM) (13). As shown in Fig. 4 A, the overexpression of Ii with or without DM had little effect on MR1 expression, but a significant effect on class II expression. To determine whether Ii and/or DM could be detected in physical association with MR1, coimmunoprecipitation experiments were performed on the above Ltk⁻ transfectants. In this experiment, Ii was precipitated with mAb In-1 or DM was precipitated with a rabbit polyclonal antiserum (28) and the respective precipitates were blotted with mAb 4E3 to detect MR1. As shown in Fig. 4 (B and C), significant amounts of MR1 coprecipitated with Ii in lysates of the A^d.gli and A^d.IiDM cell lines, and significant amounts of DM precipitated with MR1 in a lysate of the A^d.IiDM cell line. In parallel, the endogenous Ii and DM were also associated with MR1 in the mouse B cell CH27 (unpublished data). Importantly, *in vitro* mixing experiments demonstrated that these associations with MR1 did not occur after cell lysis (unpublished data). In contrast to its association with Ii and DM, MR1 was not detected in association class II (Fig. 4 C). These disparate associations are of considerable interest in light of previous findings that CD1d is coprecipitated with Ii and MHCII (29) in humans and Ii alone in mice (16, 20). It is also important to note that the tail of MR1 lacks known sorting motifs like the ones on tails of CD1 isoforms (16). Thus, MR1 may be more dependent on Ii for endosomal trafficking.

To determine whether Ii or DM might impact MAIT cell activation in a manner independent of increased surface expression, these cell lines were tested for their ability to activate MAIT cell hybridomas. As shown in Fig. 4 C, the overexpression of Ii and both Ii and DM significantly

enhanced activation of both MAIT hybridomas tested. It is intriguing that the presence of DM appears to accentuate both the physical association of MR1 with Ii, as well as the MAIT cell activation. However, overexpression of Ii alone clearly augments MAIT cell activation in these cell lines, possibly by facilitating MR1 trafficking to an endosomal compartment.

Ii causes partial redistribution of MR1 to late endocytic compartments

We demonstrated that MR1-specific activation of MAIT cells is enhanced by overexpression of Ii. It is known that Ii facilitates the transport of class II molecules to endocytic compartments (12, 13). To test whether Ii facilitates the transport of MR1 molecules from the ER to the endocytic pathway, we generated an mMR1.EGFP fusion protein by linking EGFP to the C terminus of mouse MR1 and expressed mMR1.EGFP in the cells with different Ii expression levels. Subcellular localization of MR1 showed that MR1 colocalized more with Lamp1 in cells overexpressing Ii. Pearson's coefficient of colocalization of MR1 and Lamp1 was higher in Ii-expressing cells than the parental cells (Fig. 5 A). Importantly, the percentage of MR1 that colocalized with Lamp1 protein increased in Ii-expressing cells with statistical significance (Fig. 5 B). These results indicate that Ii facilitates the partial redistribution of MR1 from the ER to late endocytic compartments.

Late endosomal compartments are critical for MR1 trafficking and MAIT cell activation

The evidence presented thus far that endosomal trafficking enhances MAIT cell activation was obtained with L cells.

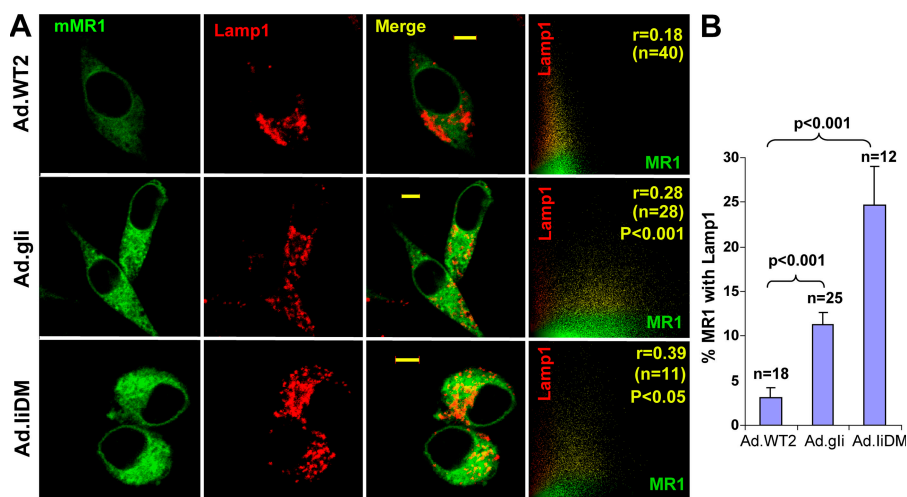


Figure 5. MR1 is partially redistributed into Lamp1⁺ compartments in cells overexpressing Ii. Ii-overexpressing cells were transduced with mMR1.EGFP and stained with rat anti-mouse Lamp1. (A) The colocalization of MR1 (green) with Lamp1 (red) are analyzed using the imaging analysis program Velocity and are plotted to the right. The *r* values for each cell type were obtained by averaging the Pearson's coefficients of colocalization from multiple cells (*n* indicates the cell number). The significance (*P*) of the *r* value was examined using a paired Student's *t* test. Bars, 5 μ m. (B) The percentage of MR1 localization in the late endocytic compartment (Lamp1⁺) was analyzed and shown to be significantly higher with Ii overexpression. Data represent the mean \pm the SEM from multiple cells (*n*). The *P* values were obtained using a paired Student's *t* test.

Although the physiological APC for MR1 antigen presentation is not known, *in vivo* studies demonstrated that B cells are required for the expansion and/or selection of MAIT cells (2). However, no detectable surface expression of MR1 was observed on isolated primary B cells or any other primary cells (unpublished data), probably because appropriate conditions for inducing MR1 cell surface expression at levels sufficient to activate MAIT cells are not known (8). To determine whether overexpression of transfected MR1 in B cell lines activates MAIT cells in a late endosome-dependent fashion, we used the vacuolar proton (H⁺) ATPase inhibitors concanamycin A (CMA) and bafilomycin A1 (Baf A1) to prevent acidification of late endocytic compartments. It should be noted that these inhibitors have been shown to interfere with antigen presentation for CD1 isoforms and class II molecules (30, 31). Surface expression of MR1 was reduced on CH27.mMR1 cells with the inhibitor CMA for 16 h, whereas surface expression of class Ia molecules was unaffected (Fig. 6 A). Furthermore, leupeptin, which is known to block Ii proteolysis and class II trafficking from the endosome to the surface (32, 33), was found to similarly reduce MR1 surface expression by retaining MR1 in the late endocytic compartments (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20072579/DC1>). More strikingly, MAIT cell stimulation was ablated by CMA treatment of MR1-expressing B cells. As a negative control, activation of the alloreactive anti-L^d hybridoma L3 (34) was not affected by CMA treatment (Fig. 6 B). Similar results were obtained using Baf A1 (Fig. S3). Reduction of MR1 surface expression and ablation of MAIT cell activation through inhibition of the vacuolar acidification strongly support the conclusion that MR1 traffics through the late endosomal/lysosomal compartments for antigen presentation.

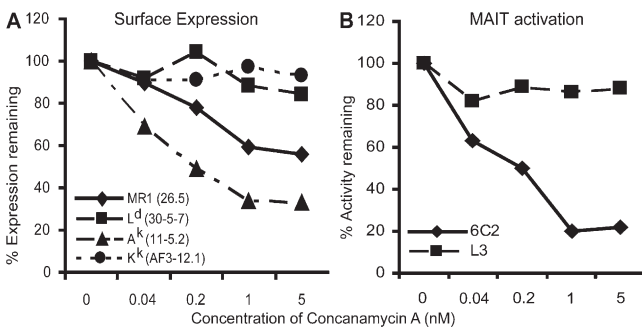


Figure 6. Inhibition of late endocytic compartments reduced MR1 surface expression and blocked MAIT activation. (A) CH27.mMR1 cells were treated with different concentrations of CMA and stained with indicated antibodies with isotype-matched irrelevant antibodies as controls. The percentage of remaining surface expression compared with the DMSO solvent group was plotted. Data represent results from five experiments. (B) MR1-expressing cells treated with CMA were co-cultured with MAIT hybridoma 6C2, or L^d-restricted hybridoma L3. The IL-2 production from CMA-treated groups was compared with the DMSO-treated group, showing as remaining activity upon inhibition. Five experiments were done with similar results.

Interference of endogenous Ii expression reduces MR1 surface expression on B cells and inhibits MAIT cell activation

We next assessed the consequences of inhibiting endogenous Ii expression in a B cell line, the putative MAIT cell APC. To this end, a small hairpin RNA (shRNA) expression vector targeting different fragments of mouse Ii was transduced into the B cell line CH27.mMR1 (Fig. 7 and Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20072579/DC1>). The Western blot shown in Fig. 7 A (and quantified in Fig. 7 B) indicated that the RNA interference significantly reduced Ii protein expression, whereas MR1 protein expression remained stable in whole-cell lysates. However, surface expression of MR1 was reduced ~40% upon inhibiting Ii expression (Fig. 7 C). By comparison, MHC class II expression was more affected by reduced Ii expression, but class I expression of K^k (not depicted) or L^d (Fig. 7 C) was unaffected. Most strikingly, reduced Ii expression led to >80% inhibition of MAIT activation (Fig. 7 D), whereas activation of the L^d reactive T hybridoma L3 was unaffected (not depicted). Importantly, the consequences of Ii depletion look strikingly similar to the aforementioned CMA treatments, in that surface expression was less affected than MAIT cell

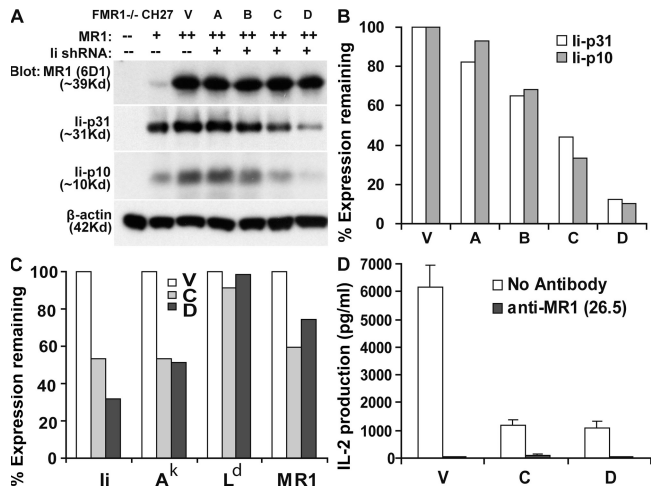


Figure 7. Interference of Ii expression reduces MR1 surface expression on B cells and depletes MAIT cell activation. CH27.mMR1 cells were transduced with different shRNA (Fig. S4) to interfere with Ii expression. (A) Western blot of Ii in shRNA-transduced CH27.mMR1 cells (A–D) is compared with vector control (V), non-MR1-transduced B cells (CH27), and MR1-deficient fibroblasts (FMR1^{-/-}). The blots shown for the major isoform p31 and cleaved fragment p10 are from two different exposures caused by differences in intensity. (B) Western blot results were quantified by the percentage of intensity of shRNA-transduced cells against the vector only. (C) With the best interference on Ii, shRNA-C- and -D-transduced CH27.mMR1 cells were stained for intracellular Ii and surface MHC molecules. Results were plotted with the percentage of MFI against vector control. (D) shRNA- and vector-transduced CH27.mMR1 cells were used to activate MAIT cell hybridoma 6C2. Blocking with anti-MR1 mAb indicated that MAIT cell activation was MR1 specific. Data are representative of two experiments, and are shown as the mean ± the SD.

activation (Fig. 6). These combined findings provide compelling evidence that surface MR1 expression is less dependent on endosomal trafficking than MAIT cell activation.

B cell–expressed MR1 mainly localizes to the ER and endocytic compartments

To visualize the MR1 trafficking pathway and further confirm the importance of the late endosomal compartment, the fusion protein mMR1.EGFP expressed in B cells was localized using confocal microscopy in the context of ER and late endocytic markers, CRT or Lamp1, respectively. Of note, the intact mMR1 molecule on CH27.mMR1 and fusion protein on CH27.mMR1.EGFP activated MAIT cells similarly (Fig. S5, A and B, available at <http://www.jem.org/cgi/content/full/jem.20072579/DC1>). Microscopy examination revealed that mMR1.EGFP mainly resided in the Lamp1⁺ late endosome/

lysosome, and the CRT⁺ ER (Fig. 8, A and B). Both of these associations were found to be statistically significant when assessed using Pearson's coefficient calculation (Fig. 8 A). ATPase inhibitor CMA-treated CH27.mMR1.EGFP cells displayed a “donutlike” topology of swelled late endocytic compartments with intensive mMR1.EGFP staining, further confirming that a major subcellular location of MR1 is in the late endocytic compartments (Fig. S6). In addition, immunoelectron microscopy demonstrated that MR1 primarily localizes to ER tubular structures (Fig. 8 C) and endocytic compartments (Fig. 8 D), especially late endosomes as exemplified by their multivesicular bodies (Fig. 8 E). MR1 localization in multivesicular endosomes corroborates its dysfunction upon blocking of endosomal acidification. These data demonstrate that MR1 traffics through and likely binds ligand in a late endocytic compartment, as shown in the schematic model (Fig. 8 F), but

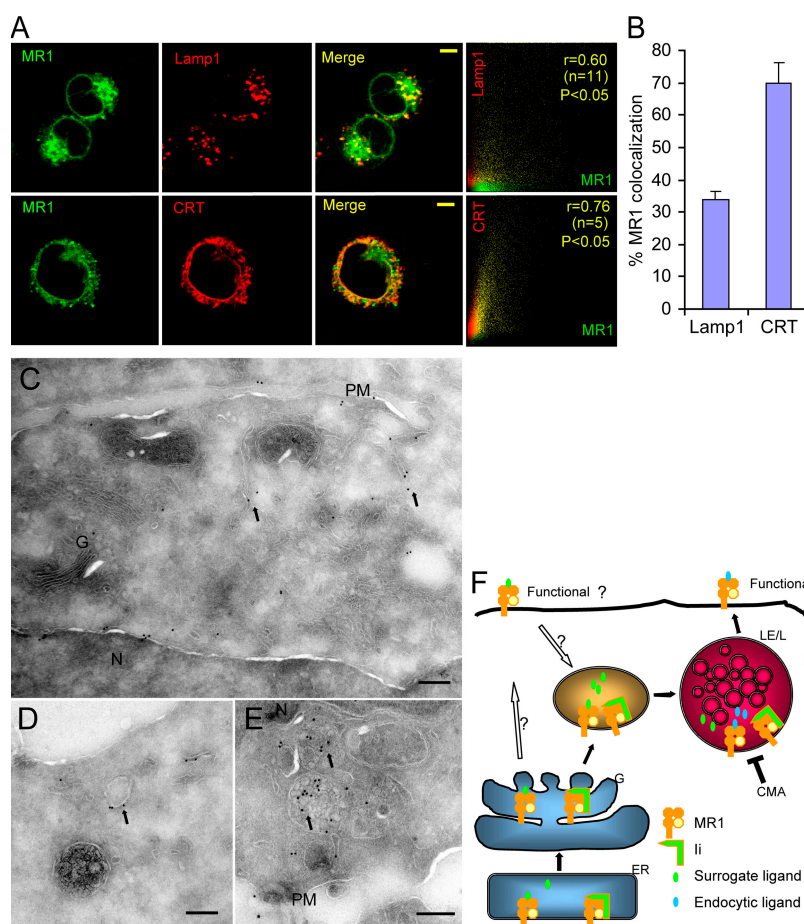


Figure 8. Late endosomal distribution of MR1 molecules in B cells visualized by confocal and cryoimmunoelectron microscopy. (A) CH27.mMR1.EGFP was stained with antibodies against Lamp1 and CRT. The quantitation of colocalization between MR1 and Lamp1 or CRT was plotted to the right. The r and P values were obtained with the calculation used in Fig. 5. Staining was repeated three times with similar results. (B) Percentage of MR1 colocalization in different compartments was plotted as the mean \pm the SEM (C) CH27.mMR1.EGFP were stained with rabbit anti-EGFP antibody visualized with 18-nm colloidal gold using electron microscopy. N, nuclear region. MR1 labeled by gold particles is shown at the tubular structures (arrow), plasma membrane (PM), or Golgi complex (G). (D) Some MR1 particles reside at the single vesicle (arrow), indicating an endosomal compartment. (E) More particles (arrow) localize in the multivesicular body (MVB) representing the late endosome compartments. (F) Schematic model of MR1 endocytic trafficking. MR1 egresses from the ER with either a surrogate ligand or association of li. The li facilitates MR1 traffic to late endocytic compartments to obtain a hypothetical ligand and further express onto the cell surface for MAIT cell activation. Bars: (A) 5 μ m; (C–E) 200 nm.

do not exclude the possibility of a direct surface expression for the activation of other MAIT cell clones.

DISCUSSION

In this study, we present evidence that the class Ib molecule MR1 presents a ubiquitously expressed endogenous ligand that can activate MAIT cells via an endocytic pathway. Supporting this notion, MAIT cell activation by MR1 does not require the MHC class I peptide loading complex, but is facilitated by the MHC class II chaperone Ii for endocytic trafficking. This finding is important in the context of studies of MHCII and CD1 isoforms, because binding of ligands in different endocytic compartments allows presentation of various endogenous or microbial ligands to $\alpha\beta$ T cells that consequently mount appropriate regulatory or effector responses.

Although the nature of the MR1 ligand is not known, there is considerable evidence that presentation of an endogenous ligand is involved in MAIT cell activation. This evidence includes the following: (a) MR1 undergoes a conformational change from open to folded analogous to MHC molecules after binding a ligand (35–37), and only antibodies to the folded MR1 conformation block MAIT cell activation; (b) extensive mutagenesis of the MR1 predicts that it interacts with ligand in a manner similar to classical MHC molecules (6); and (c) low molecular weight components isolated from precipitated MR1 are able to enhance MAIT cell activation in an MR1-dependent manner (unpublished data). Furthermore, the endogenous ligand presented by MR1 is ubiquitously expressed, as suggested by the observation that disparate cell lines overexpressing MR1 can activate select MAIT cell hybridomas in a manner independent of exogenous antigens. That the endogenous ligand presented by MR1 is able to activate MAIT cell hybridomas, but not primary MAIT cells, probably results from insufficient presentation or lack of adequate costimulatory signals on the transfectants. Similarly, in the absence of ligand, primary *i*NKT cells only respond to cells expressing very high levels of CD1d (17, 30). Therefore, we used MAIT cell hybridomas to study the pathway of endogenous antigen presentation. Indeed, studies of the CD1d endogenous ligand *i*Gb3 clearly set a precedent for the presentation of an endogenous ligand in the context of a bacterial infection (20, 38). Extending this analogy, MR1, like CD1d, might also be capable of direct presentation of an exogenous ligand of presumed gut flora origin.

From previous studies of MHCI, MHCII, and CD1 isoforms, it is clear that molecular chaperones play key roles in determining intracellular trafficking, and thus their respective ligand acquisition. Unlike classical class Ia molecules, MR1 expression and MAIT cell activation *in vitro* do not require proteasome function, TAP transporter, or the ER molecular chaperones tapasin and CRT. The lack of TAP dependency is consistent with the observation that the *in vivo* MAIT cell development is also TAP independent (1, 2). In regard to the presentation pathway used by MR1, these findings suggest that MR1 does not require a peptide processed by the proteasome, or translocated into the ER by TAP. Therefore, if MR1 does bind a peptide, its processing and binding likely

occur outside of the ER because the proteasome–TAP pathway is the predominant provider of ER peptides for class I molecules (24). It is also possible that association with the class I peptide loading complex could alter MR1 ligand binding and activation of other MAIT cell clones, in a manner similar to CD1 molecules that use both ER loading of a lipid ligand to activate $V\alpha 14^+$ NKT cells, and endosomal sampling for $V\alpha 14^+$ *i*NKT activation (1, 2). Alternatively, MR1 could bind a nonpeptide ligand such as a glycolipid. Indeed, Shimamura et al. reported that α GalCer-related synthetic glycolipids, α -mannosyl ceramide activates MAIT cells from a TCR transgenic mouse in what appeared to be an MR1-dependent manner (9, 10). However, this observation has been difficult to reproduce with MAIT cell hybridomas used here (unpublished data). Structural comparisons of the MR1 α/β domain sequences superimposed on the ligand binding platforms of class Ia and Ib molecules predict that if MR1 does bind a peptide or a glycolipid, it does so using a unique anchoring scheme not shared with class Ia or CD1 proteins, respectively (8).

Unexpectedly, MR1 was found to use a pathway with similarities to class II molecules to enable efficient MAIT cell activation. For example, inhibition of vacuolar acidification reduced MR1 expression to $\sim 60\%$ of untreated controls. This reduction was less than that seen with class II, but dramatically different than class Ia molecules, which were not affected by inhibition. More strikingly, MAIT cell activation was profoundly blocked by vacuolar H^+ ATPase inhibitors, to an extent not simply explained by decreased MR1 surface expression. For example, from previous studies we know that the 60% of maximal surface expression is sufficient to provide full MAIT cell activation (6). These combined findings suggest that surface expression of MR1 can be attained without endosomal trafficking; however, activation of these MAIT cell hybridomas requires endosomal trafficking likely to bind a ligand. Furthermore, confocal studies suggest that MR1 localizes in Lamp1⁺ late endocytic compartments, similar to class II molecules. This localization was further confirmed via immunoelectron microscopy by visualization of multivesicular endosomes containing a significant number of MR1 particles. Thus, like class II proteins, MR1 potentially samples ligands in endocytic compartments for MAIT cell activation.

Further support that MR1 uses an endocytic pathway for presentation is that MR1 is associated with Ii and this association facilitates its endosomal localization. Importantly overexpression of MR1 with Ii greatly enhances MAIT cell activation. Reciprocally, interference of endogenous Ii expression in B cells, the presumed physiological mucosal APC, abrogated MAIT cell activation. These results strongly suggest that Ii chaperoning of MR1 facilitates its transport to endocytic compartments, which presumably contain the ligands required for activation of these MAIT cell hybridomas. This model is supported by the known role of Ii in promoting transport of class II through endocytic compartments to sample peptide ligands (12, 13). However, for certain class II ligands, Ii association is not required (13, 39). Accordingly, a reduced number

of CD4⁺ T cells develop in Ii-deficient mice, and interestingly, they display a more self-reactive phenotype (39, 40).

Ii also facilitates antigen presentation by certain CD1-lipid complexes. Although Ii promotes endosomal trafficking and ligand acquisition by the CD1d isoform, this role is redundant with sorting sequences in its cytosolic tails (16). For example, Ii is able to enhance V α 14⁺ iNKT cell activation by tailless CD1d molecules, and Ii-deficient cells exhibit impaired activation of V α 14⁺ iNKT cells, but not V α 14⁻ NKT cells. Interestingly, activation of V α 14⁺ iNKT requires an endosome-derived ligand, whereas V α 14⁻ NKT cells are activated by tailless CD1d that are likely loaded a ligand through the secretory pathway (16, 20). Similarly, Ii appears to play a prominent role in the presentation of certain, but not all MR1 ligands. This conclusion is based on our observation the lack of Ii expression has only a minimal effect on MR1 surface expression, but a profound effect of MAIT hybridoma cell activation. Furthermore, like with iNKT cells, MAIT cell development is Ii independent (3). However, analogous to the development of CD4⁺ T cells, the absence of Ii could alter the ligand specificity of developing MAIT cells, skewing it toward increased self-reactivity. Indeed, consistent with this speculation, the self-reactive MAIT cell hybridomas characterized in this study were isolated from Ii- (and TAP-)deficient mice. In contrast, our attempts thus far using MR1 overexpressing cell lines to stimulate primary MAIT cells or hybridomas generated from the V α 19i transgenic mice have been negative (unpublished data). But regardless of whether the MR1 ligand is from an endogenous or exogenous source, the studies here demonstrate that MR1 can present ligand from an endocytic compartment to MAIT cells.

Physiological models proposed to explain MR1 function can now incorporate the fact that MR1 traffics through an endocytic compartment. It has been shown that MAIT cell development is dependent on the presence of MR1, commensal flora, and the BCR J_H chain, but neither B1 B cells, the μ chain, nor isotype switching (21). A likely candidate for mediating MAIT cell expansion is B2 B cells, especially the subset expressing IgA in the gut (21). It could be envisioned that MR1 presentation to MAIT cells may be a negative feedback loop to temper IgA secretion (2, 21). Upon endocytosis of microbial components during bacterial translocation from the gut lumen into submucosal tissues, B cells start producing IgA and inhibiting bacterial translocation. This process is exemplified in the μ MT mice, which harbor IgA-producing B cells and have a normal MAIT cell population (21, 41). MR1 expressed by B cells may have the potential to load endogenous ligands in endosomal compartments induced by the commensal bacteria. Alternatively, MR1 may also be capable of directly binding a gut bacteria-specific ligand processed in an endocytic compartment. Either of these models is consistent with the hypothesized interaction between these B cells and MAIT cells for gut immune regulation or MAIT cell development (2, 21). The fact that primary MAIT cells from V α 19i transgenic mice rapidly secrete cytokines, including IL-4, -5, and -10, and up-regulate ICOS expression indicate that MAIT cells can initiate Th2-like or inhibitory

responses upon TCR ligation (4). Indeed, MR1 inhibiting the pathogenesis in an EAE model provides the first evidence for its potential regulatory role in autoimmune responses (5). The endocytic pathway used by MR1 for antigen presentation likely contributes to the secretion of both stimulating and inhibitory cytokines by MAIT cells, similar to the cytokine production by different subsets of NKT cells (42) or CD4⁺ T cells. Regardless of the physiological function of MAIT cells, the ability of MR1 to sample endogenous or induced-endogenous ligands is likely an integral factor determining how MAIT cells control the balance of homeostatic versus autoimmune responses.

MATERIALS AND METHODS

Cell lines. Mouse B6 (H2b) embryonic fibroblast WT3, and fibroblasts deficient for TAP1 (FT1⁻ or TAP^{-/-}), tapasin (Tpn^{-/-}), CRT (K42 or CRT^{-/-}), or K^b/D^b (KODO) were used for transduction (23). Ltk⁻ transfected with I-A^d (A^d.WT2), A^d and genomic Ii sequence (A^d.gli), or A^d genomic Ii sequence, and H-2DM α and β chains (A^d.IiDM) have been previously described (13). Mouse B cell line CH27 (H-2^a) (43) was a gift from P. Allen (Washington University, St. Louis, MO). MAIT T-T hybridoma cells, 6C2, and 8D12, have been described previously (1, 6). CD8⁺ T hybridoma (L3) (34) specifically reactive to H-2L^d molecule was a gift from O. Kanagawa (Washington University, St. Louis, MO). We generated MR1-deficient embryonic fibroblast FMR1^{-/-} from MR1^{-/-} mice (2). All cells were maintained as previously described (6).

Gene cloning and retroviral transduction. Generation of the mMR1 and mMR1/L^d molecules was previously reported (6, 22). Fusion protein mMR1.EGFP was generated using three-piece ligation of PCR-amplified mMR1, and EGFP genes with the vector pMX.IRES.Puromycin modified from pMX retroviral expression system (44). Retrovirus-containing supernatants were generated and used for transduction as previously described (6).

Antibodies. Anti-mMR1 mAb 12.2, 26.5, and 4E3 were previously described (22), as were mAb B8-24-3 (anti-Kb; American Type Culture Collection), mAb 30-5-7 (anti-Ld) L^d (45), and mAb K24-199 (I-A^d) (13). The new anti-MR1 mAb 6D1 (unpublished data) was used for Western blot. mAb 34-2-12 (anti-D^d), PE-labeled 11-5.2 (I-A^b), FITC-labeled AF3-12.1 (anti-K^b), mAb 34-5-3 (I-A^d), In-1 (anti-mouse Ii), biotinylated mIgG2a, and Fc receptor blocker were purchased from BD Biosciences. Rabbit anti-H-2DM was used for immunoprecipitations and blotting, as previously described (28). Mouse anti- β -actin AC-74 (Sigma-Aldrich) was used as a loading control in Western blots. For confocal microscopy, rat anti-mouse Lamp1 1D4B (IgG2a) was purchased from BD Bioscience, and rabbit anti-CRT SPA-600 was obtained from Stressgen. Alexa fluor-conjugated secondary antibodies were purchased from Invitrogen.

Proteasome or lysosome inhibition. Proteasome inhibitors lactacystin (Alexis), NLYS (26), drug BFA (Epicentre), and vacuolar H⁺ ATPase inhibitors CMA (Calbiochem) and bafilomycin A1 (Sigma-Aldrich) (30, 31) were used to treat MR1-expressing cells for 16 h.

Flow cytometry. The MR1-transduced fibroblasts were analyzed using a FACSCalibur (BD Biosciences) as previously reported (6). B cells were treated with Fc receptor blocker before applying biotinylated 26.5 or 30-5-7 with biotinylated mIgG2a as an isotype control. Intracellular staining of Ii was performed by using biotinylated In-1 antibody after fixing cells with 2% paraformaldehyde and permeabilizing with 1% saponin.

MAIT cell activation. 10⁵ mMR-expressing cells/ml were co-cultured with 10⁶/ml MAIT hybridoma cells for 24 h, and the secreted IL-2 was

tested as previously described (6). Purified anti-mMR1 mAb was used to block MAIT activation at 10 $\mu\text{g}/\text{ml}$. The recombinant mouse IL-2 (Bio-source) was used to generate a standard curve.

Immunoprecipitations and Western blots. As previously described (22), we used anti-MR1 antibody mAb 26.5 coupled with protein A-Sepharose 6MB (Sigma-Aldrich) to precipitate mMR1 and anti-Ii (In-1) bound with GammaBind G-Sepharose (GE Healthcare) to precipitate mouse Ii. For Western blots, MR1 was detected by mAb 4E3 or 6D1 as previously described (6), whereas Ii was stained by biotinylated In-1. Results of Western blot were quantified using ImageJ software (National Institutes of Health).

RNA interference of Ii molecule. The retroviral shRNA expression vector pSRPU6 vector was modified from pSUPER.retro.puro (OligoEngine) by replacing the H1 promoter with the human U6 promoter. This newly constructed vector was tested with high efficiency for inducing small interference RNA (siRNA) to block GFP expression. CH27 cells transduced with mMR1 were further infected with retrovirus expressing siRNA as selected in the Fig. S4. Infected cells were screened with 4 $\mu\text{g}/\text{ml}$ puromycin and tested with Western blot, FACS, and MAIT cell activation 3–7 d after antibiotics selection.

Confocal microscopy and image analyses. CH27.mMR1.EGFP cells were grown on Laboratory-Tek chambered coverglass slides (Nalge Nunc), stained with anti-Lamp1 or CRT antibodies, and observed using LSM510 META Laser Scanning Confocal Microscope (Carl Zeiss, Inc.) as previously reported (46). Negative control used isotype matching irrelevant rat or mouse mAbs or purified preimmune rabbit serum. Images were processed with LSM image browser and analyzed with the software Volocity 4.0 (Improvision). Colocalization of MR1 with Lamp1 and CRT molecules was analyzed by testing each pixel for the presence of both green (mMR1.EGFP) and red colors under the same threshold for multiple cells. Pearson's coefficient (r) for each cell was calculated by Volocity (47), and the significance of this coefficient was tested with the sample size. The percentage of colocalization of MR1 with Lamp1 or CRT was calculated with measurement module from the Volocity, and the paired Student's t test was used to examine the significance of difference between groups.

Cryoimmuno electron microscopy. CH27.mMR1.EGFP cells were fixed in 4% paraformaldehyde (Polysciences, Inc.) and 0.01% glutaraldehyde (Polysciences, Inc.). Ultrathin sections were stained with rabbit anti-EGFP antibody and followed by the goat anti-rabbit IgG conjugated to 18-nm gold (Jackson ImmunoResearch Laboratories) as previously reported (46). Staining with the secondary conjugate only and the CH27.mMR1 were used as negative controls. Staining was observed with the JEOL 1200EX transmission electron microscope (JEOL USA).

Online supplemental material. Fig. S1 shows that the MR1 protein is ubiquitously expressed in different mouse cell lines and tissues, as determined by the immunoprecipitation and Western blot. Fig. S2 shows that treatment of cells with leupeptin down-regulates the MR1 surface expression, impairs Ii processing, and retains MR1 in late endocytic compartments. Similar to findings shown in Fig. 6 using CMA, Fig. S3 shows that another H^+ ATPase inhibitor, bafilomycin A1, reduces MR1 surface expression and inhibits MAIT cell activation. Fig. S4 shows the siRNA sequences used for interfering with endogenous Ii expression. The data shown in Fig. S5 demonstrates that adding EGFP to the C terminus of MR1 protein does not impact on its surface expression or ability to activate MAIT cells. Fig. S6 shows swollen late endocytic compartments with MR1 accumulation upon applying the inhibitor CMA. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20072579/DC1>.

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