Evidence for MR1 Antigen Presentation to Mucosal-associated Invariant T Cells*

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Shouxiong Huang‡, Susan Gilfillan‡, Marina Cella‡, Michael J. Miley‡, Olivier Lantz§, Lonnie Lybarger‡, David H. Fremont‡, and Ted H. Hansen‡

From the ‡Department of Pathology and Immunology, Washington University, St. Louis, Missouri 63110, §Laboratoire d’Immunologie and INSERM U520, XIInstitut Curie, 26 rue d’Ulm, Paris, France, and ¶Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, Tucson, Arizona 85724-5044

The novel class Ib molecule MR1 is highly conserved in mammals, particularly in its α1/α2 domains. Recent studies demonstrated that MR1 expression is required for development and expansion of a small population of T cells expressing an invariant T cell receptor (TCR) α chain called mucosal-associated invariant T (MAIT) cells. Despite these intriguing properties it has been difficult to determine whether MR1 expression and MAIT cell recognition is ligand-dependent. To address these outstanding questions, monoclonal antibodies were produced in MR1 knock-out mice immunized with recombinant MR1 protein, and a series of MR1 mutations were generated at sites previously shown to disrupt the ability of class Ia molecules to bind peptide or TCR. Here we show that 1) MR1 molecules are detected by monoclonal antibodies in either an open or folded conformation that correlates precisely with peptide-induced conformational changes in class Ia molecules, 2) only the folded MR1 conformer activated 2/2 MAIT hybridoma cells tested, 3) the pattern of MAIT cell activation by the MR1 mutants implies the MR1/TCR orientation is strikingly similar to published major histocompatibility complex/αβ TCR engagements, 4) all the MR1 mutations tested and found to severely reduce surface expression of folded molecules were located in the putative ligand binding groove, and 5) certain groove mutants of MR1 that are highly expressed on the cell surface disrupt MAIT cell activation. These combined data strongly support the conclusion that MR1 has an antigen presentation function.

Classical MHC class I (or class Ib) proteins are highly polymorphic, expressed on all nucleated cells, and have well-defined peptide presentation functions (1). By comparison, non-classical class I (or class Ib) typically have more limited polymorphism, more restricted tissue expression, and more diverse functions (2, 3). Interestingly, however, certain class Ib proteins have specialized antigen presentation functions such as human HLA-E and mouse Qa-1 that present signal peptides of other class I molecules to T cells or NK cells (2). Alternatively, mouse and human CD1 present glycolipid ligands to T or NK-T cells (4). However, other class Ib molecules such as HFE and ZAG have non-immunological functions, and still others like MR1 have unknown functions. Despite these functional differences, sequence comparisons and recent crystallographic studies indicate that class Ib proteins are structurally very similar to class Ia proteins (5–7).

Lymphocytes with restricted repertoires such as B1 B cells, some γδT cells, and NK-T cells that express autoreactive, invariant antigen receptors have been called innate lymphocytes (8). The restricted repertoires of these cells may allow them to rapidly respond to phylogenetically conserved antigens (9). Alternatively, lymphocytes with restricted repertoires may have regulatory roles dependent upon the recognition of self-ligands. Until recently, NK-T cells were the only known T cell subset with an invariant TCRs conserved between mouse and man, perhaps reflecting an ancient and important physiological function (9, 10). More specifically, most mouse NK-T cells express an invariant TCRα V-J junction (Va14-Jα18) with a CDR3 of constant length paired with limited Vβ segments (11, 12). Unlike conventional T cells, their development is not altered in TAP−/− mice. Consistent with this, NK-T cells recognize glycolipids such as α-galactosyl ceramide presented by the MHC class Ib molecule CD1d. It is noteworthy, however, that the endogenous ligand presented physiologically by CD1d was difficult to identify (13, 14). NK-T cells are thought to bridge innate and adaptive immune responses by secreting large amounts of cytokines, particularly IL-4, upon stimulation, and NK-T cells have been implicated in T cell polarization, tumor rejection, and autoimmunity (15–17).

A second type of T cell with an invariant TCR was recently defined and, because they preferentially home to the gut mucosa, were named MAIT (mucosal-associated invariant T) cells (18, 19). Like most NK-T cells, MAIT cells express a TCRα chain encoded by a specific Vα-Jα rearrangement with a CDR3 segment of constant length and minor sequence diversity that preferentially pairs with a limited number of Vβ segments (9). More specifically, the canonical sequence of the MAIT cell TCR (iVβ7.2-Jα19) is encoded by hVα7.2-Jα33 in humans and the highly homologous mVα19-Jα33 in mouse and cattle. MAIT cells reside within the CD4 CD8 T population in human, mice, and cattle as well as the CD8α− subset in humans (18). Like NK-T cells, MAIT cells are selected on hematopoietic cells in a TAP-independent, β2m-dependent manner. Also similar to
NF-T cells, MAIT cells are selected/activated by a class 1b molecule. As recently shown by Treiner et al. (19), the development and activation of MAIT cells is dependent on the mono-morphic class I-related molecule MR1, which is remarkably conserved among mammals (indeed more conserved than CD1d) (20–22). In addition to MR1, MAIT cell development is dependent upon B cells and commensal flora, properties not shared with NK-T cells (19, 23). In regard to tissue distribution, MAIT cells accumulate in the mucosal system, whereas NK-T cells are abundant in internal organs like the spleen and liver (9).

Properties of MAIT cells unique or shared with NK-T cells have led to speculation about MAIT cell function (9). For example, based on their preferential accumulation in the gut mucosa, it was proposed that MAIT cells could contribute to the discrimination between pathogens and commensals or be involved in a negative feedback loop to regulate IgA secretion, which plays a critical role in controlling the gut microbial flora. MAIT cells might also interact with dendritic cells, which are abundant in the mucosal lamina propria and are critical for antigen presentation during bacterial infections (24). Thus, resolving MAIT cell function could provide key insights into how the immune system maintains the balance between tolerance and immune responses in mucosal tissues.

Formidable obstacles must be overcome to test the validity of any hypotheses regarding MAIT cell function. MAIT cells are rare and represent around 2% of CD4+ CD8- lymph node T cells but are somewhat more abundant in the intestinal mucosa of B6 mice. So far attempts have failed to generate MAIT cell clones from primary cells, but fortunately, MAIT T-T hybridomas were obtained for functional studies (9, 18). A number of MAIT hybridomas were selected based on their expression of the TCR and were obtained for functional studies (9, 18). In addition to difficulties in obtaining MAIT cells, MR1 poses its own challenges for investigation. Most notably, endogenous expression of MR1 has not been defined, and thus far there is no direct evidence that MR1 binds a ligand and, if so, what its chemical nature might be.

Cells transfected or transduced with mMR1 cDNA were shown to express low levels of MR1 on the surface; however, most MR1 protein remained intracellular (3). These findings are consistent with, but not evidential of ligand limiting MR1 expression. Furthermore, the low level of MR1 protein detected on the cell surface after transfection/transduction was augmented by swapping the α3 domain of mMR1 with that of the classical class I molecule H-2Ld (3). Biochemical characterization of insect cell expressed recombinant MR1 protein demonstrated stoichiometric association with β2m and provided evidence for N-linked glycosylation analogous to that found in all class Ia molecules (3). These MR1 expression studies used either (i) anti-epitope tag mAb 64-3-7 that is specific for “open” (not associated with ligand) forms as shown with class Ia molecules and H2-M3 (25–28) or (ii) mAb 4E3 generated in β2m-deficient mice immunized with a peptide derived from the α2 domain of mMR1. Given this, we questioned whether ligand associated or folded MR1 was missed in our detection system. Indeed, a soluble ectodomain of recombinant MR1 was secreted by insect cells, suggesting that it was folded. And this recombinant MR1 was detected by mAb 64-3-7 only after denaturation, consistent with MR1 folding into a 64-3-7 negative conformer, analogous to class Ia molecules when they undergo peptide-induced folding (9). However, we have thus far been unable to define a conventional peptide ligand bound to recombinant MR1. Related to this uncertainty regarding an MR1 ligand is the question of whether MAIT cell activation is ligand-dependent.

To better characterize MR1 function in inducing MAIT activation and the possible requirement for a potential ligand, we generated new mAbs against MR1 by immunizing MR1 knockout mice with recombinant MR1 protein. Importantly these new mAbs to MR1 detected a folded conformer of MR1 missed in previous investigations. These new mAbs were used to show that only folded MR1 activated both MAIT cells tested. Furthermore, a panel of MR1 mutants were used to (i) indicate that the TCR of MAIT cells engages MR1 in a very similar orientation as that previously reported for β2TCR/MHC engagements and (ii) make predictions about how MR1 interacts with a putative ligand that controls its expression and MAIT cell activation.

EXPERIMENTAL PROCEDURES

Cell Lines—The B6 (H-2b) embryonic fibroblast WT3 (29) and TAP1-deficient line (25, 30) were used for retroviral transduction. Mouse MR1-transfected WT3 (WT3.mMR1) was previously described (3) as were the isolation and characterization of MAIT T-T hybridoma cells 6C2 and 8D12 (18). The IL-2-dependent cell line CTL-2 (31) was used to test IL-2 secreted by the hybridomas. All cells were maintained in RPMI 1640 or DMEM (Invitrogen) media supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.25 mM HEPES, and 100 units/ml penicillin/streptomycin.

Gene Cloning and Retroviral Transduction—A bicistronic retroviral vector (pMSCV.IRES.Neomycin, pMIN) encodes the gene of interest from the upstream cistron and antibiotic resistance gene from the downstream cistron (32). Generation of the epitope-tagged mMR1 and the chimeric mMR1/Ld molecule (mMR1 (α1-α2 domain)/Ld (α3-cyto)plasm) was previously reported (3). Genus encoding mMR1 and mMR1/Ld in the vector pRES.neo (3) were cut with restriction enzymes and inserted into the multicloning sites of retroviral vector pMIN. The inserted gene was confirmed with the BigDye terminator cycle sequenc- ing kit (ABI, Foster City, CA). Retrovirus-containing supernatants were generated using the Vpack vector system (Stratagene, La Jolla, CA) for transient transfection of 293T cells (33) to produce ectropic virus for the infection of WT3 cells. Packaging cells were transfected using Fu-GENE 6 (Roche Applied Science). Virus-containing supernatants were collected 48–72 h post transfection, and 2–3 ml was added to target cells (∼106) per infection. Cells were selected and maintained under 0.6 mg/ml Geneticin (Invitrogen) or 0.3 mg/ml hygromycin (Sigma) post transduction. These transduced cells were maintained as a single population with stable expression of the targeted protein for more than 6 months in culture.

mAb—mAb 4E3 was produced in β2m-deficient mice immunized against a peptide corresponding to MR1 residues 130–153 (3). mAb 64-3-7 is an epitope tag specific for open forms of class I molecules (27). mAb B6-23-4-3 (ATCC, Manassas, VA) detects folded H-2Kb, and mAb B8–24-3 (ATCC, Manassas, VA) detects folded H-2Ld. To produce new mAbs to MR1, MR1-deficient mice (19) were immunized with the soluble ectodomain of insect-expressed human MR1/β2m complexes (3). Hybridomas were generated by fusing splenocytes with Sp2/0 cells, and supernatants were initially screened by enzyme-linked immunosorbent assay on plates coated with either hMR1/hβ2m or hβ2m alone. Those clones that recognized hMR1/β2m but not hβ2m were then screened by flow cytometry using transfectants expressing hMR1 or mMR1 (3).

Sequential Immunoprecipitation and Western Blots—For sequential immunoprecipitation, mMR1-transfected WT3 cells (107) were lysed in phosphate-buffered saline, pH 7.4, with 1.0% digitonin (Wako, Richmond, VA), 20 mM iodoacetamide (Sigma), 0.2 mM phenylmethylsulfonyl fluoride (Roche Applied Science) for 1 h on ice. Non-transfected WT3 cells were used as a control. Saturating amounts of anti-MR1 mAbs 12.2, 26.5, 4E3, or epitope tag mAb 64-3-7 were incubated with protein G-Sepharose 4 fast flow (Amersham Biosciences) for 2 h at 4°C with rocking. The anti-Kb mAb B8–24-3 was used as a negative control for pre-clearance. After washing with phosphate-buffered saline twice, antibody-bound protein G was incubated with post-nuclear lysates for 4 h. To assure complete removal of reactive MR1 molecules the supernatants were subjected to a second pre-clearance with protein G bound by the same antibodies. For the
RESULTS

**mAbs Detect Two Distinct MR1 Conformers, Analogous to the Ligand Empty (Open) and Ligand-associated (Folded) Class Ia Molecules**—We previously reported low levels of surface expression of MR1 by transfection or transduction using the epitope tag mAb 64-3-7 that is specific for non-ligand-associated class Ia molecules where it has been established that classical class I molecules such as Kb and Kd have higher percentages of folded conformers indicative of better overall ligand binding (26–28,35). More specifically, nascent class I heavy chains transition from an open conformer to a folded conformer when they bind a high affinity peptide in the endoplasmic reticulum, and reciprocally, surface class I heavy chains transition from a folded conformer to an open conformer after peptide dissociation (36). Thus, it was of interest to determine whether this paradigm also applied to MR1. Open conformers in these aforementioned studies were detected by mAb 64-3-7, and folded conformers were detected by conformation-dependent, allele-specific mAbs. To determine whether MR1 also exists as alternative open versus folded conformers, sequential immunoprecipitation experiments were performed using mAb 64-3-7 and the new conformational-dependent mAbs to MR1. A lysate of WT3.mMR1 cells was precleared with a negative control antibody (B8–24–3), the epitope tag mAb 64-3-7, or mMR1-reactive mAbs 12.2 and 26.5. Supernatants from each of these pre- cleared antigen preparations were then tested with the same or alternative mAb. As shown in Fig. 1B the pattern of reactivity in the sequential immunoprecipitation experiment was striking and defined two MR1 conformers, 64-3-7− (12.2, 26.5−) and 64-3-7+ (12.2, 26.5)+. Evidence that both mAbs 12.2 and 26.5 detect the same conformer was demonstrated by the fact that they cleared for each other. Although the apparent lower affinity of mAb 4 precluded its use as a clearance reagent, mAb 4-reactive MR1 proteins were removed by both 12.2 and 26.5 but not 64-3-7 (data not shown). Therefore, all three new anti-MR1 mAbs 12.2, 26.5, and 4 are conformation-dependent and detect an MR1 conformer distinct from mAb 64-3-7. Given that these new mAbs were raised against folded MR1 protein and the clear parallel findings with class Ia molecules and H2-M3 (26–28, 35), it was concluded that mAbs 12.2, 26.5, and 4 all detect the folded MR1 conformer.

**Both Open and Folded Conformers of MR1 Are Expressed on the Cell Surface but Only Folded MR1 Activates MAIT Cells**—Given that the new mAbs 12.2, 26.5, and 4 detect a conformer of MR1 previously not detected by mAbs 4E3 and 64-3-7, it was of interest to reevaluate MR1 surface expression. As shown in Fig. 2A, mMR1-transduced cells express low levels of open (64-3-7−) MR1 as previously reported. However, these same cells expressed substantially higher levels of folded (12.2+) MR1. Comparable staining was observed with mAb 26.5 (data not shown). Interestingly, higher levels of surface expression were obtained by transduction with a chimeric gene consisting of the mMR1 α1/2 domains connected to the α3 transmembrane and cytoplasmic domains of L3 (3) (Fig. 2A). Like native MR1, the chimeric molecule was detected as a folded conformer at higher levels than as an open conformer. It is of interest to compare these findings with conformers of L3, a class Ia protein that is a relatively poor peptide binder (37). Intact MR1, chimeric MR1/L3, and intact L3 have a steady state surface expression of 70–90% folded conformer. By contrast, other class Ia molecules such as Kk and Kβ have higher percentages of folded conformers indicative of better overall ligand binding (28). In any case, surface MR1 is detected as alternative conformers similar to class Ia molecules where it has been established that the open conformer arises after ligand dissociation (26).

The existence of alternative conformers of MR1 on the cell surface raised the interesting question of which conformer activates MAIT cells. To address this question, different anti-MR1-reactive mAbs were used to block MAIT cell activation. Two MAIT cell hybridomas, namely 8D12 and 6C2, were used for virus production and transduction of WT3 cells. Flow Cytometry—For surface staining, 10^6 cells per sample were incubated on ice in microtiter plates with a saturating concentration of mAb. After washing, phycoerythrin-conjugated goat anti-mouse IgG (BD Pharmingen) was used to visualize the primary antibody staining. Intracellular MR1 molecules were stained with fluorescein isothiocyanate-conjugated mAb 64-3-7 as described (3). Flow cytometric analyses were performed using a FACSCalibur (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences).

MAIT Cell Activation and mAb Blocking—Biological activity of IL-2 secreted by MAIT T-cell hybridoma lines 8D12 and 6C2 was used to estimate the degree of MAIT cell activation. 10^5/ml mMR1/Ld-transduced WT3 cells were cultured with 10^5/ml hybridoma cells in complete RPMI 1640 medium. Purified anti-mMR1 mAbs were added at different concentrations. Supernatants were harvested and frozen at −80 °C for at least 1 h to lyse trace cells that may have carried-over. CTL2 cells were washed and added to supernatants at a final cell density of 5 × 10^6/200 μl/well in a 96-well plate. Triplicate wells were run for each sample. CTL2 cells in the absence of culture supernatant were used as the negative control, and the addition of serial IL-2 dilutions was used as positive control. After 18–24 h of incubation, Alamar blue (Bio-Tek Instruments, Winooski, VT) was added at 20 μl/well, and relative amounts of IL-2 in each supernatant were determined by fluorescence on a multi-detection microplate reader (Bio-Tek instruments).
in the current report (18, 19). WT3 cells expressing either intact mMR1 or the mMR1/Ld were found to activate both 8D12 and 6C2 MAIT hybridomas (not shown). However, the cells expressing the mMR1/Ld chimeric molecule were consistently better stimulators of MAIT cells and, therefore, were used for this antibody blocking study. As shown in Fig. 2B, mAbs 12.2 and 26.5 displayed a dose-dependent inhibition of MAIT cell activation resulting in complete blockage at 10 μg/ml. By contrast, neither mAb 4E3 or 64-3-7 inhibited MAIT cell activation (Fig. 2B). Similar findings were seen with both MAIT hybridomas in multiple assays. Thus, the failure of mAb to the open MR1 to block MAIT cell activation and more importantly the complete blocking by mAb to the folded MR1 indicate that only the folded MR1 conformer activates these two MAIT cell hybridomas.

**Mutagenesis Strategy**—The high degree of sequence similarity of MR1 with classical class Ia proteins (>40% identity in α1/α2) readily enabled us to design a structure-based mutagenesis strategy to investigate the antigen presentation function of MR1. To identify sequences interacting with TCR or putative ligand, we mutated 23 residues in the α1/α2 domains of MR1. These residues are at positions corresponding with ones previously implicated in class Ia interaction with peptide or TCR by mutagenesis or crystallographic studies (38–49) (Fig. 3A). Most residues (17 of 23) were replaced with amino acids located in the corresponding positions in class Ia molecules so as not to disrupt overall structures. Four MR1 mutations (Y7L, H59A, W159A, F171A) were made at positions where all class Ia heavy chains have invariant tyrosines involved in χ pocket anchoring of the peptide N terminus (50). And three MR1

**Fig. 1.** mAbs to mMR1 detect two alternative mMR1 conformers. A, new mAbs were generated to recombinant hMR1 in MR1 knock-out mice, and their species specificity was determined by testing on cells expressing either hMR1 (upper panels) or mMR1 (lower panels). Of the eight mAbs that displayed strong staining with MR1, five were hMR1 specific, whereas three mAbs (designated 4, 12.2, and 26.5) cross-reacted strongly with mMR1. Profiles representative of each type of new mAb (shaded) are shown with isotype control antibodies (unshaded). B, sequential precipitation was performed by preclearing a cell lysate of WT3.mMR1 with mAbs listed along the left side of the figure. Each precleared lysate was then tested with mAbs listed along the top. Eluted samples were run on SDS-PAGE and blotted with biotinylated mAb 64-3-7 under denaturing conditions to detect MR1. mAb B8–24-3 (anti-folded K*) was used as a negative control mAb (Ctl) for preclearance. Similar sequential precipitations were performed multiple times with consistent findings.
mutations (H80T, H84A, A146K) were made at positions where class Ia molecules have conserved threonine, tyrosine, or lysine residues, respectively, involved in F pocket anchoring of the peptide C terminus (50). Each of the 23 MR1 mutants was expressed by transduction as a MR1/Ld chimeric molecule in WT3 cells to achieve the highest level of functional MR1 expression. Cells expressing each mutant were then tested for surface MR1 expression and its ability to activate MAIT cell hybridomas.

**MR1 Mutants Predict a TCR Engagement Similar to That of MHC/Peptide**—To identify residues of MR1 that are required for MAIT cell activation, MR1 mutants in helical residues with high levels of surface expression of folded molecules were considered relevant based on our above findings. Fortunately we had three mAbs for these analyses, mitigating problems of mutations affecting antibody detection. Indeed, as noted in Fig. 3, B and C, mutant Y155R ablated detection of MR1 by mAbs 12.2 and 26.5 but not by mAb 4. Thus, to assess expression of

![Fig. 2. Surface expression of and MAIT cells activation by open and folded MR1 conformers.](image-url)

**A.** % folded for mMR1, mMR1/Ld, and Ld. B6/WT3 cells transduced with mMR1/Ld, mMR1, and Ld were stained with mAb 64-3-7 (epitope tag specific for open forms) as shown in the upper panels. Alternatively mAb 12.2 (folded MR1) or mAb 30–5-7 (folded Ld) was used for staining cells shown in the lower panels. % folded is a relative comparison based on the calculation folded/folded + open × 100. Negative control (thin line) represents secondary reagent only (comparable findings were also obtained using an isotype-matched negative control mAb). Mean fluorescent intensity (MFI) minus the negative control is shown above each peak. Surface expression was tested numerous times and remained constant. B, the mMR1/Ld-transduced WT3 cells were incubated with MAIT T-T hybridoma line 6C2 for 24 h with or without different concentration of listed mAbs. The amount of IL-2 in samples was determined using IL-2-dependent CTLL-2 cells, and fluorescence of alamar blue product was used to quantify the amount of proliferation. Percent activity was determined after subtracting the background of CTLL-2 cells alone and then dividing by readings of samples without antibody. Background levels caused by non-transduced WT3 cells without additional antibody are indicated as a thin line. Similar findings were obtained using MAIT cell line 8D12.
FIG. 3. Effects of mutations on mMR1 surface expression and MAIT activation. A, the predicted MR1 structure is imposed upon a template of the Kα α1/α2 domains (81) with the accommodations of minor deletions (red) and additions (blue). MR1 mutations characterized here are indicated by purple spheres (helical residues), green spheres (β-sheet residues), and yellow spheres (terminal ligand anchor residues). B, analyses of MFI of mMR1 mutants. Residues are shown as the same color scheme in Fig. A. MFI of mMR1 surface expression is normalized on negative controls, and the ratio to wild type (MFI_{mutant}/MFI_{wildtype}) is shown. Shaded areas highlight folded mMR1 with the ratio to wild type < 0.5. C, surface expression of seven of the MR1 mutants (red line) compared with wild type (green line). The black line is the negative control and represents the secondary reagent only. Consistent findings were seen in several different assays. Note all MR1 mutants have similar levels of expression of MR1 as detected by conformation-independent mAb 4E3. This finding supports the fact that retrovirus expression results in comparable levels of expression. By contrast, all mutants shown except R9V and A149Q displayed significant differences as detected by the conformation-dependent mAbs 12.2, 26.5, and 4. As discussed, these differences are important for mapping epitopes and defining the role of a
folded MR1, we considered mutations detected at >60% of the wild type level by at least one of the mAbs to folded MR1. This level of expression was determined in functional assays to be well above the level required for MAIT cell activation (data not shown). As expected from studies of class Ia molecules, most MR1 mutations in alleged helical residues (13/16) were expressed at high levels as folded MR1 proteins. The three exceptions were MR1 mutations H59A, W159A, and F171A, all located at positions in class Ia molecules involved in anchoring of the N terminus of the peptide. Thus, to avoid ambiguities between peptide binding and TCR contacts, we excluded all MR1 positions where class Ia molecules have residues involved in terminal peptide binding (these are considered as a group in the last section of “Results”). Of the remaining 11 MR1 mutants in helical residues, 4 (L66K, G69R, A166E, and Y155R) ablated activation of both MAIT cell hybridomas, and another mutant (K75R) sharply reduced activation of both MAIT cell hybridomas (Fig. 4A). Furthermore, mutations A76V and Q82R activated the 6C2 hybridoma significantly better than the 8D12 hybridoma, suggesting either a subtle difference between these two hybridomas in affinity for, or orientation with, MR1. In any case, these MR1 mutations that ablate MAIT cell activation are at positions previously shown in mutagenesis studies of several different mouse and human class Ia molecules to affect TCR interaction. Although co-crystallographic studies have demonstrated some variation in the orientation that TCR engages MHC/peptide, the MR1 mutations that ablate MAIT cell activation clearly reside within the collective TCR interfaces (Fig. 3D). More specifically, the TCR mutagenesis footprint on MR1 is concordant with the co-crystallographic footprint of the N15 or 2C TCRs bound to Kb/cognate peptides (39, 48). This finding strongly suggests that the α1/α2 antigen binding platform of MR1 has an analogous interaction with TCR as MHC-peptide complexes (38–49).

putative MR1 ligand in the expression of a folded MR1 conformer. D, MR1 mutants predict TCR orientation and the role of ligand in MAIT cell activation. Location of mMR1 mutants that affect MAIT activation are shown on the H-2Kb surface-accessible model (generated using software GRASP) (81). The pink indicates helical mutations with surface expression >60% but not activating MAIT cells (the mutant K75R may slightly activate 6C2), except that mutant A76V and Q82R display the different degree in activating two MAIT cell lines. The green area designates sites of groove mutations expressed as folded molecules at the surface at levels that are sufficiently high enough to activate MAIT cells but that do not. Enclosed by the dotted line is an area that represents TCR binding area. The line is drawn around the Ca atoms of the 4 TCR contacting the MHC residues, which have been previously defined (38–43, 45).

Fig. 4. MAIT cell activation by MR1 mutants. A, MAIT hybridoma cell activation by mMR1 mutations of residues in the presumed helical regions of the α1/α2 domains. B, MAIT interaction with mMR1 mutations corresponding to residues involved in the ligand binding by class Ia molecules. Only MR1 mutants expressed at >0.6 of wild type are shown to obviate concerns of low level expression precluding MAIT cell activation. Accordingly, mutants F22Y, Y95I, and L114Q are not shown. For both panels WT3 cells expressing the indicated mutant were incubated with MAIT T-T hybridoma line 8D12 (upper) or 6C2 (lower) for 24/48 h with or without mAbs 12.2, 26.5, or an isotype control mAb 34-2-12 (anti-H-2Dk). Relative IL-2 production was determined by proliferation of CTLL-2 cells as monitored by fluorescence of alamar blue product (y axis). The base line indicates the background level of MAIT activity when co-culturing with non-transduced WT3 cells. The S.D. represents triplicates in an alamar blue assay. None signifies no antibody.
MR1 Mutants Located in the Putative Ligand Binding Groove Affect MAIT Cell Activation and Expression of Folded MR1 Conformers—We next assessed whether mutations in the putative MR1 ligand binding groove affected MAIT cell activation. Again, only mutants expressed at >60% of the wild type level were evaluated. Of the seven mutations made in the MR1 putative ligand binding groove (Y7L, R9V, F22Y, Y95I, R97E, F113Y, and L114Q) only four (Y7L, R9V, R97E, F113Y) were expressed at a sufficiently high levels for MAIT cell testing. As shown in Fig. 4B, mutations Y7L and R97E ablated activation of both MAIT cell hybridomas, and F113Y was detected by both hybridomas. Interestingly, R9V ablated activation of the 6C2 hybridoma but strongly activated the 8D12 hybridoma. Thus, these substitutions at MR1 positions 7, 9, and 97 allowed folded MR1 conformers to be expressed but dramatically affected MAIT cell activation. As noted in Fig. 3D, residues 7, 9, and 97 are predicted to be clustered in the center of the putative MR1 ligand binding groove, well within the predicted interface with the TCR. Drawing parallels with similar mutagenesis studies of class Ia molecules (51–54), it is attractive to speculate that residues 7, 9, and 97 are involved in MR1 ligand positioning or ligand discrimination.

Of the seven mutations discussed above in the putative MR1 ligand binding groove, three (F22Y, Y95I, and L114Q) had less than half the levels of folded molecules at the cell surface as detected by all three mAbs to folded MR1. However, all three of these mutations had levels of open MR1 comparable with wild type as detected by mAbs 64-3-7 or 4E3. It is noteworthy that class Ia mutagenesis studies have implicated each of these residues as dramatically affecting peptide binding (55–60). Based on these parallels, the phenotype of these three mutants is suggestive of overall poor ligand binding and a requirement for ligand occupancy to retain a folded conformation.

As shown in Fig. 3B there are some interesting inconsistencies between MR1 detection by mAbs 12.2 and 26.5 versus mAb 4. As noted earlier, Y155R ablated detection of mAbs 12.2 and 26.5 but not 4. This strongly suggests that residue 155 is part of the 12.2 and 26.5 epitope but not the 4 epitope. And mutations W159A and F171A sharply reduce detection by all three mAbs, suggesting they may also contribute to each epitope. However, it cannot be ruled out that these latter mutations more profoundly prevent MR1 from attaining a folded conformation. Perhaps most intriguing of the serologic differences was the observation that mAb 4 is clearly more sensitive to perturbation in the putative ligand binding groove than mAbs 12.2 and 26.5. For example, Y95I, R97E, and L144Q all sharply reduced mAb 4 staining compared with mAb 12.2 and 26.5 staining. There are now several examples of mAbs made against Ia or class II molecules loaded with endogenous ligands that display some ligand discrimination (61–64). Thus, a possible explanation of this finding is that reactivity of mAb 4 with MR1 proteins have two properties that predict that they have a conserved function of physiological relevance. First mMR1 and hMR1 share 90% identity in their α1α2 domains that far exceeds the 70% or less identity shared by these regions of mouse and human class Ia and Ib proteins (20, 21). Second, MR1 is the activation and restriction element for a population of T cells preferentially expressed in the gut lamina propria with an invariant CDR3α called MAIT cells (19).

Extending these findings, in this study we report multiple lines of evidence supporting the model that MR1 functions as a unique antigen presentation molecule with properties shared with both class Ia and class Ib molecules. Key to this conclusion, several findings reported here provide compelling circumstantial evidence that MR1 binds a ligand that determines the expression of folded MR1 proteins as well as the activation of MAIT cells. For example, 1) MR1 is detected in an open versus folded conformation analogous to class Ia molecules when they bind peptide, 2) certain MR1 groove mutations known to affect class Ia peptide binding impair the expression of folded MR1 protein, 3) only the folded MR1 conformer activates MAIT cells based on 2/2 hybridomas tested, 4) MR1 engages the TCR in an orientation strikingly similar to αβTCR engagements of MHC/peptide complexes as implied by MAIT cell activation by our panel of MR1 mutants, and 5) certain MR1 groove mutations with high surface expression levels affect MAIT cell activation. Further supporting the role of a ligand in MR1 folding, we were unable to refold recombinant MR1 after bacterial expression using folding conditions that yielded folded non-ligand binding class Ib proteins T10, T22, MICA, MICB, RAE-1 (3, 67–71). However, a folded recombinant ectodomain of MR1 was secreted by insect cells in the presence of highly supplemented media. Thus, accumulating evidence supports the notion that MR1

**DISCUSSION**

MR1 proteins have two properties that predict that they have a conserved function of physiological relevance. First mMR1 and hMR1 share 90% identity in their α1α2 domains that far exceeds the 70% or less identity shared by these regions of mouse and human class Ia and Ib proteins (20, 21). Second, MR1 is the activation and restriction element for a population of T cells preferentially expressed in the gut lamina propria with an invariant CDR3α called MAIT cells (19). Extending these findings, in this study we report multiple lines of evidence supporting the model that MR1 functions as a unique antigen presentation molecule with properties shared with both class Ia and class Ib molecules. Key to this conclusion, several findings reported here provide compelling circumstantial evidence that MR1 binds a ligand that determines the expression of folded MR1 proteins as well as the activation of MAIT cells. For example, 1) MR1 is detected in an open versus folded conformation analogous to class Ia molecules when they bind peptide, 2) certain MR1 groove mutations known to affect class Ia peptide binding impair the expression of folded MR1 protein, 3) only the folded MR1 conformer activates MAIT cells based on 2/2 hybridomas tested, 4) MR1 engages the TCR in an orientation strikingly similar to αβTCR engagements of MHC/peptide complexes as implied by MAIT cell activation by our panel of MR1 mutants, and 5) certain MR1 groove mutations with high surface expression levels affect MAIT cell activation. Further supporting the role of a ligand in MR1 folding, we were unable to refold recombinant MR1 after bacterial expression using folding conditions that yielded folded non-ligand binding class Ib proteins T10, T22, MICA, MICB, RAE-1 (3, 67–71). However, a folded recombinant ectodomain of MR1 was secreted by insect cells in the presence of highly supplemented media. Thus, accumulating evidence supports the notion that MR1
folding and MAIT cell activation are both ligand-dependent.

Despite the above-listed similarities between MR1 and class Ia proteins, there are significant differences. Whereas class Ia proteins are ubiquitously expressed, this is certainly not the case for MR1. Indeed, endogenous expression of MR1 has yet to be identified despite considerable effort on our part. Furthermore, unlike the abundance of T cells that are activated and restricted by class Ia molecules, MAIT cells are very rare, particularly in mice (9, 18). However, MAIT cells are abundant when compared with the number of antigen-specific, class Ia-restricted T cells. As noted earlier, several properties of MR1 mirror those of CD1d proteins. Most notably, like MR1 detection by MAIT cells, CD1d is detected by a unique population of T cells with an invariant TCR, namely NK-T cells. However, there are also important differences between MAIT cells and NK-T cells, including their tissue distribution and the unique dependence of MAIT cells on gut flora and B cells (9, 19).

All αβ T cells described thus far detect MHC/peptide or CD1d-lipid complexes (12, 72, 73). Therefore, MR1 would be the exception to this rule if it does not bind a ligand involved in αβ T cell activation. However, it is certainly worthwhile considering this possibility. Relevant to this issue are studies of T22, a non-ligand binding class Ib protein detected by γδ T cells (68, 74). Indeed, a mutagenesis study of the groove of class Ib molecule T22 was interpreted as evidence that it binds a ligand in a similar manner as class Ia molecules (74). Subsequent crystal structure analysis, however, showed T22 does not bind a ligand and has a severe truncation of its α2 helix exposing its β-sheet floor for direct contact with the γδ TCR (68). The γδ TCR has extruded CDR3 loops (75). Thus, T22 groove residues are potential direct contacts for γδ TCR interaction. However, this is clearly not the case for MR1 groove mutations. Unlike T22, MR1 is predicted to have intact α1 and β2 domains that appear to engage the TCR in a manner strikingly similar to MHC/peptide (Fig. 3D), and the CDR3s of MAIT TCR are of normal length (18). Furthermore if MR1 does not bind a ligand required for stable surface expression and MAIT cell function, then one might expect its groove would need to close in a manner analogous to other ligand-independent MHC molecules. However, if this were the case one would expect a different set of solvent-exposed residues to protrude from the MHC helices and be involved in MAIT cell activation. Because this was not found, our data suggest that MR1 makes direct contact only with helical and not groove residues in a manner analo-
with αβTCR engagements with MHC/peptide.

Difficulty in defining endogenous expression of MR1 has been interpreted as evidence that MR1 may be inducible. Thus, in light of findings reported here it is attractive to speculate that a limiting self-ligand, whereas its physiological function is to present bacterial-de-
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