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Review

# How MR1 Presents a Pathogen Metabolic Signature to Mucosal-Associated Invariant T (MAIT) Cells

Hamish E.G. McWilliam[1] and Jose A. Villadangos[1,2,*]

**Mucosal-associated invariant T (MAIT) cells are innate-like lymphocytes restricted by the antigen (Ag)-presenting molecule MHC class I (MHC I)-related protein 1 (MR1). The Ags presented by MR1 are vitamin B-related Ags (VitBAgs), 'building-block' metabolites of riboflavin that are synthesized by a range of microbes. MR1 presentation is thus a unique mechanism for the immune detection of a pathogen metabolic signature. While the full picture of how MR1 accomplishes this remains incomplete, recent data show that, unlike other MHC molecules, MR1 operates by a presentation-on-demand mechanism. In the absence of metabolite ligands MR1 is mostly stored in the endoplasmic reticulum (ER). Ligand binding leads to the formation of a Schiff-base bond between MR1 and its ligand, triggering a 'molecular switch' in MR1 that allows trafficking of the complexes to the cell surface. The complexes are subsequently internalized and mostly degraded irrespective of the affinity of the interaction between MR1 and its ligands. Here we review past and recent studies that have contributed to defining this pathway and propose new directions for a full understanding of the role and mechanisms of MR1 Ag presentation.**

## MR1 Presents Derivatives of a Vitamin Building-Block Metabolite Conserved in Microbes

Multicellular organisms have evolved sophisticated mechanisms to fight infection by distinguishing self from foreign. The innate immune system uses multiple devices to directly recognize molecules that are fundamental 'building blocks' common to many pathogens —pathogen-associated molecular patterns (PAMPs) — allowing innate immune cells to respond rapidly to broad groups of organisms [1]. In contrast to this rapid and broadly specific system of pathogen detection, the adaptive immune system relies on vast numbers of B and T lymphocytes, which differ in the expression of Ag receptor variants that are highly specific for unique motifs. In the case of classical T cells, each of their individual receptors (TCRs) recognizes a small number of peptides bound to MHC molecules displayed on the surface of Ag-presenting cells (APCs). The combined specificity of receptors expressed by all T cells ensures that virtually every pathogen-specific peptide — and hence the protein from which the peptide is derived — can be recognized by some member of the T cell repertoire, but the low

### Trends

Innate-like T cells can respond quickly to conserved antigens from diverse pathogen classes presented on MHC-like proteins and thus share characteristics with cells of the adaptive and innate immune systems.

MHC class I-related protein 1 (MR1) presents a unique class of antigens to the abundant innate-like mucosal-associated invariant T (MAIT) cell population: MR1 displays 'building-block' metabolites derived from the synthesis of vitamin B2 (riboflavin), allowing the mammalian immune system to detect a pathogenic metabolic signature.

MR1 surface presentation is tightly regulated by the metabolite ligands themselves and is characterized by a 'presentation-on-demand' mechanism. MR1 loads these metabolites inside the endoplasmic reticulum, which triggers a 'molecular switch' in MR1 that allows trafficking to the plasma membrane, after which the complexes are degraded irrespective of the affinity of the interaction between MR1 and its ligand.

Other locations for MR1 loading may include the cell surface and intracellular compartments, although their relative contributions to MR1 presentation remain uncertain.

The putative molecular machinery involved in ligand handling, MR1 stabilization, complex formation, and regulation of MR1 trafficking and fate are

frequency of each T cell means a potent response against any particular infection requires several days to develop [2].

Sitting between these two extremes of speed versus specificity landscape are semi-invariant T cells, which have attributes of both the innate and the adaptive immune system. Semi-invariant T cells are relatively abundant and express TCRs with limited variability, so the detection of and initiation of responses against pathogens that produce the Ag recognized by their TCR can be very rapid [2]. Such Ags are generally not peptides but non-protein structural components that are presented by non-classical MHC molecules; for example, CD1d molecules present lipid Ag [3] to invariant natural killer T (*i*NKT) cells. The list of Ag types recognized by semi-invariant T cells was further expanded in 2012 to include VitBAgs, when it was revealed that these ligands are presented by MR1 to MAIT cells [4]. The MR1–MAIT cell axis represents a novel system for the detection of infection based on the recognition of the metabolic signature, rather than the structural components, of pathogens. As metabolites can be released in the extracellular environment, detection of this signature may not require a physical encounter between the pathogen and the APC that stimulates MAIT cells. In this review we recapitulate the history of MR1 research, summarize recent knowledge on the MR1 Ag presentation pathway that has been possible to acquire with the identification and availability of VitBAgs, and propose future directions for a full understanding of the role of the MR1–MAIT cell axis in the immune system.

high-priority objectives for the MR1–MAIT cell field and represent potential targets for immunomodulation.

## A Short History of the Discovery of the MR1–MAIT Cell Ag Presentation Axis

The *Mr1* gene was initially discovered in 1995 using a polymerase chain reaction-based strategy to amplify genes with sequence similarity to the α3 domain of MHC I [5]. Unlike MHC I, the *Mr1* gene is monomorphic and located outside the MHC region. MR1 is highly conserved among species [6], particularly in the regions encoding the α1 and α2 domains, which form the Ag-binding site of classical MHC I and CD1 molecules. Not all MHC-like molecules have Ag-presenting functions, but this structural conservation between distant species suggested that if MR1 did present Ag this would also be highly conserved and evolutionarily important for host defense and survival [7,8].

Evidence gradually emerged showing that the novel MR1 did indeed have Ag-presenting characteristics: it bound $\beta_2$-microglobulin ($\beta_2$m) – the second component of MHC I and CD1 molecules, associated with members of the peptide-loading complex [9,10] – and adopted MHC I-like 'open' and 'closed' structural conformations [11,12]. These early studies also noted that MR1 exhibited limited surface expression and remained sensitive to endoglycosidase H [10], an enzyme that cleaves carbohydrates from glycoproteins that have not yet trafficked through the Golgi, suggesting that MR1 resides in the ER. This implied that MR1 expression on the cell surface might depend on the availability of an unknown ligand, reminiscent of the mouse H2-M3 molecule, which is expressed on the cell surface only when it binds *N*-formylated peptides derived from bacteria or mitochondria [13,14].

Concurrent with these emerging studies on MR1, a new population of semi-invariant T cells conserved in mammals was described. After Porcelli *et al*. [15] described the presence of T cell populations with conserved TCRα chains in multiple human donors, the Lantz group showed that one of these invariant T cell populations was likely to be restricted by an unknown MHC Ib molecule whose expression required $\beta_2$m but not transporter associated with Ag processing (TAP), a critical component of the MHC I presentation pathway [16]. In a landmark publication, this cell population now designated MAIT cells was shown to be MR1 restricted [8]. Another salient observation made in this study was that whereas MAIT cells were constitutively produced in the thymus, their expansion in peripheral tissues required MR1 recognition in the presence of microbes [8]. While this suggested that MR1 displays microbial Ags, it was also

[1]Department of Microbiology and Immunology, Peter Doherty Institute of Infection and Immunity, The University of Melbourne, Parkville, Australia
[2]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Australia

*Correspondence: j.villadangos@unimelb.edu.au (J.A. Villadangos).

speculated that microbes were simply providing bystander signals and that the Ag might be an endogenous ligand [17] or that MR1 was recognized by itself, devoid of ligand or bound to a ligand that did not directly participate in recognition by the MAIT TCR [7,8]. Further characterization revealed a high degree of interspecies crossreactivity between MR1 and the MAIT TCR, to the extent that mouse, rat, and bovine MR1 can activate human MAIT cells [7,18]. This evolutionary conservation suggested an important function for the MR1–MAIT cell axis, but further studies into potential roles were hampered for almost another decade until the elusive MR1 ligands were unequivocally identified.

## The Road to MR1 Ag Discovery: Leads, Detours, and Success

The path to discovery of MR1 ligands opened with the realization that the ectodomain of MR1 could be secreted as a stable heterodimer with $\beta_2$m only when expressed in cell-culture media supplemented with yeast extract and amino acids [10]. Without chaperones MHC I molecules are stable only when bound to peptide ligand [19], so it was inferred that a component of the culture medium was likely to bind to, and stabilize, MR1 in this experimental system. The same group showed that a low-molecular-weight putative ligand could be acid eluted from recombinant, stable MR1, or from immunoprecipitated cellular MR1, and that the eluate enhanced MAIT cell activation when presented by MR1 from diverse mammalian species [7]. These observations launched a search for ligands that bound to MR1 molecules and contributed to recognition by the MAIT TCR.

Two studies then showed that MAIT cells specifically respond to diverse microbial infections [18,20]. They showed MAIT cells were activated by cells exposed to Gram-positive and −negative bacterial species (such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, and *Escherichia coli*) and yeast (*Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata*). However, activation was not universal among microbes because some bacteria did not activate MAIT cells (*Listeria monocytogenes*, *Enterococcus faecalis*, and *Streptococcus pyogenes* [18,20]). Therefore, the molecular microbial product presented by MR1 appeared to be highly conserved among many bacterial and yeast species but absent from others. Interestingly, it was noted that fixed cells expressing MR1 and exposed to bacteria could activate MAIT cells, indicating that the ligand was released by bacteria and did not require host-cell processing, in critical contrast to the classical MHC I and II Ag presentation pathways [18]. Proposed Ags included the CD1 ligand glycolipid $\alpha$-manosylceramide [21], which was later discarded by other groups [17,18], and a component of the bacterial cell wall present in *M. tuberculosis* [20], which appeared to be protein derived because it was destroyed by proteolysis.

A groundbreaking paper by Kjer-Nielsen *et al*. [22] provided in 2012 the most compelling evidence of a true MR1 ligand. Unexpectedly, this was not a peptide or lipid Ag but a small, vitamin B9-related metabolite; namely, 6-formylpterin (6-FP) [22]. This molecule is spontaneously generated by degradation of folic acid, a basic constituent of RPMI-1640 culture medium [22] (Figure 1). Since then, other pterins that also bind to MR1 have been described; for instance, acetyl-6-formylpterin (Ac-6-FP), which is a more stable, acetylated analog of 6-FP [23–25]. Although these molecules bind to and induce MR1 surface expression they do not cause MAIT cell activation, implying the MAIT TCR recognizes a different, activating ligand. Such ligands were identified in *Salmonella* culture medium and were initially suggested to be metabolites derived from the riboflavin synthesis pathway (vitamin B2 related) known as ribityl lumazines [22]. Subsequently, highly potent MAIT-activating ligands were identified as the pyrimidine compounds 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) [26]. Both 5-OP-RU and 5-OE-RU are generated when the riboflavin precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) from various microbes reacts spontaneously with the ubiquitous small molecule methylglyoxal or glyoxal,

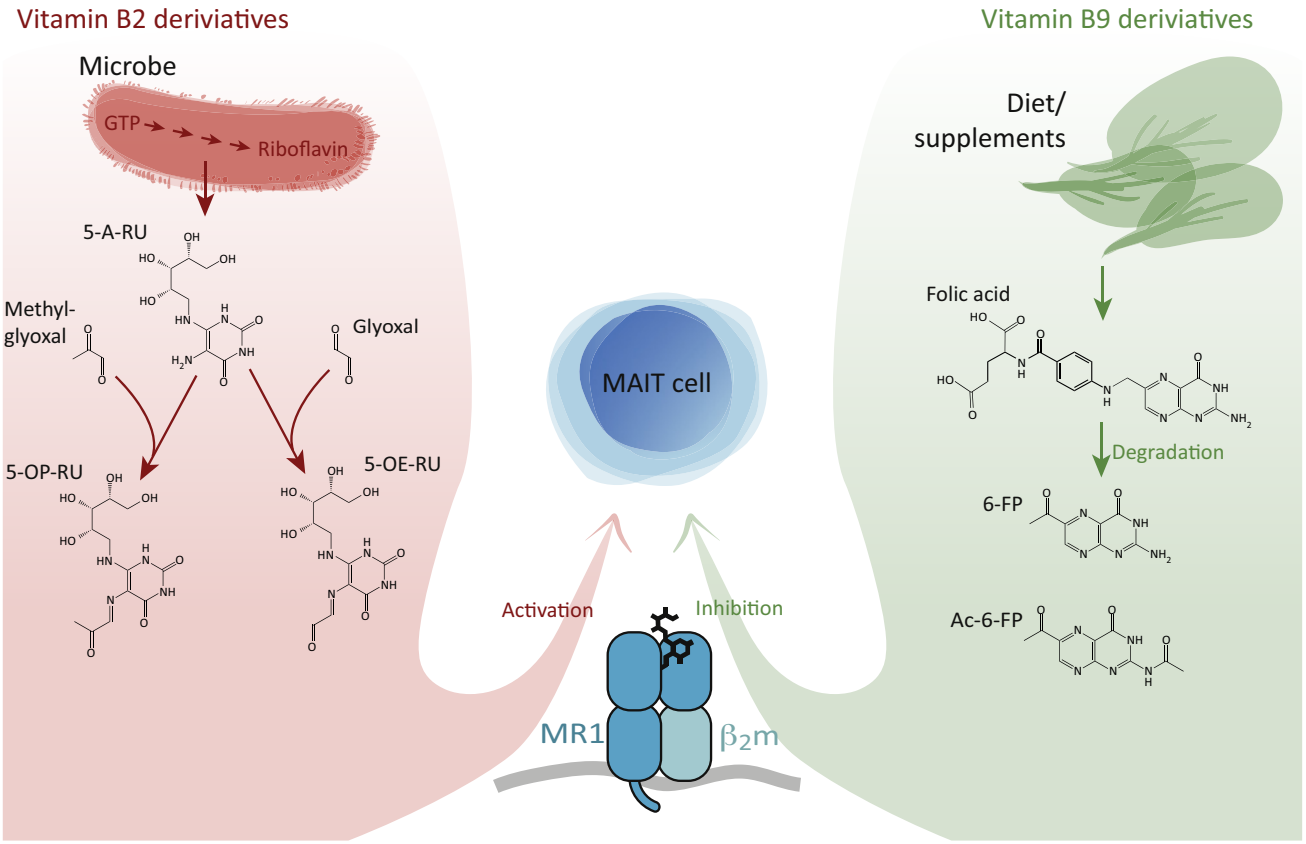


Figure 1. The Two Major Classes of MHC Class I (MHC I)-Related Protein 1 (MR1)-Binding Metabolite Ligands and Their Sources. The activating metabolite antigens (left) are generated during the synthesis of vitamin B2 (riboflavin) inside many, diverse microbes such as bacteria (red). An intermediate building block of riboflavin, 5-amino-6-D-ribitylaminouracil (5-A-RU), can be secreted by microbes and reacts with either methylglyoxal or glyoxal to form the transient antigen 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) or 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), respectively. These are captured and presented by MR1 (center) to activate mucosal-associated invariant T (MAIT) cells. The non-activating ligands are generated from the degradation of vitamin B9 (folic acid), which is acquired in the diet (green), and do not activate but antagonize MAIT cell effector function.

which may originate from the pathogen or the host (Figure 1) [26]. The single-ring compounds 5-OP-RU and 5-OE-RU are highly unstable and quickly condense to form bicyclic ribityl lumazines, but are stabilized within the MR1 binding cleft [27,28]. The weak MAIT-activating activity of synthetic ribityl lumazines has been attributed to trace amounts of 5-OP-RU [26], which is significantly more potent (see [29] for a more thorough review on this topic). In summary, these studies have described two broad categories of vitamin B-related MR1 ligands (Figure 1): the non-activating vitamin B9-related pteridines, which inhibit MAIT cell activation by functioning as antagonists and competing with the activating VitBAg for the MR1 cleft; and the MAIT cell-activating vitamin B2-related Ag derived from the riboflavin synthesis pathway, which is present in all MAIT-stimulatory bacteria and yeast and absent from those that do not activate MAIT cells [22]. A unique property of these two categories of ligands is their ability to form a covalent bond with their Ag-presenting molecule, specifically a Schiff-base bond with a lysine residue (K43) in the MR1 binding cleft (Figure 1). As discussed below, formation of this bond is critical for MR1 folding, trafficking, and, ultimately, Ag presentation.

The characterization of natural MR1 ligands has enabled the design and synthesis of more stable MR1-binding ligands capable of activating MAIT cells *in vivo* [28] as well as the discovery of drugs with related structures that can modulate the MR1–MAIT cell axis [30]. These are

exciting advances that raise the prospect of using common drugs or new synthetic ligands to modulate the MR1–MAIT cell axis *in vivo*.

## The MR1 Ag Presentation Pathway

The location where MR1 binds Ag, the intracellular trafficking route followed by the molecules before and after ligand binding, and the fate of the MR1–Ag complexes following exposure on the cell surface for MAIT cell recognition have been the subject of intense research that has sometimes yielded controversial results. Clarification of some of the discrepancies awaits further studies, but in the following sections we attempt to summarize the main conclusions in this area and speculate on potential explanations for differing observations.

### MR1 Trafficking in the Absence of Defined Ligands

Characterization of the MR1 presentation pathway was hampered before the discovery of MR1 ligands. In the presence of VitBAg the intracellular MR1 pool dramatically translocates to the cell surface [22–24,26,31]; hence, once these ligands were described it became possible to compare the intracellular behavior of MR1 before and after an Ag encounter. Earlier studies could only analyze MR1 molecules that were devoid of ligands or bound to the as-yet-uncharacterized ligands that might be present in cell-culture media [24,31,32]. Nevertheless, these studies provided useful information on MR1 trafficking that set the stage for more recent developments.

It was soon discovered that the MR1 Ag presentation pathway was distinct to both the MHC I and the MHC II pathway as it did not require proteasomes or TAP – two molecules critical for MHC I presentation – or the MHC II chaperone invariant chain (Ii) [8,17,18,33]. Despite the fact that MR1 associates with members of the MHC I peptide-loading complex [10], Huang *et al*. [17] also found that chaperones involved in class I presentation, such as tapasin and calreticulin, were likewise dispensable. However, and in contradiction to earlier work, they suggested that Ii and another MHC II chaperone, HLA-DM, were involved in MR1 presentation. They found that these proteins associated with MR1, induced MR1 localization in late endosomes, and increased MAIT cell hybridoma activation, consistent with a MHC II-like trafficking route. However, this study had three potential caveats. First, the intermolecular associations described were observed in cells overexpressing MR1, Ii, and HLA-DM, which may have caused interactions that do not normally occur in cells expressing physiological levels. Furthermore, Ii overexpression can cause dramatic changes in endosomal architecture and protein trafficking [34], which may have indirectly influenced the intracellular localization of MR1. Second, while MR1 localization in endosomal (Lamp1$^{+}$) compartments could be demonstrated, the predominant site where the molecule resided was the ER [17], suggesting an Ag presentation pathway distinct from the MHC II pathway. Third, MR1 presentation and the activation of MAIT cell hybridomas were conducted in the absence of specific Ags or bacterial infection, so it is likely that the MAIT cell clones studied were reactive against MR1 itself [35] or recognized MR1 presenting a ubiquitous folic acid derivative contained in cell culture media such as 6-FP [22]. This explanation is supported by the recent description of small numbers of autoreactive or 6-FP-reactive MAIT cells [36], which might have been the source of the hybridomas.

The description of multiple cell types equipped with a MR1 Ag-presenting function has provided additional insights into the molecular machinery associated with this pathway. The following have been shown to be capable of MR1-dependent activation of MAIT cells in the presence of bacteria (and hence VitBAg): primary human monocytes and mouse peritoneal macrophages [18,24]; monocyte-derived dendritic cells (DCs) [20]; the human B cell line C1R [24] and monocyte line THP1 [37,38]; primary human biliary epithelial cells [38]; and various fibroblast and epithelial cell lines such as the mouse cell line WT3, the human cervical carcinoma line HeLa

[18,39], and the airway epithelial cell lines A549 and BEAS2B [20,31]. This confirms the promiscuous expression and functional presentation of MR1 and suggests that the presence of chaperones specific to professional APCs such as Ii and HLA-DM is not essential for MR1 presentation.

In conclusion, while the early studies conducted with undefined MR1 ligands opened insightful lines of enquiry into MR1 trafficking in the absence of pathogens, their conclusions may not be applicable to the physiological function of the molecule in the presentation of microbial Ags. Two recent studies revisited the intracellular localization of MR1 before and after exposure of cells to microbial Ags and concluded that in the former situation MR1 was almost exclusively within the ER [24] or in both the ER and endosomes [31].

Further studies are required to clarify the intracellular trafficking route of MR1 in the absence of Ag, but the consensus is that in these conditions very little MR1 trafficks to the plasma membrane, with the majority being retained in the ER, endosomes, or both. This conclusion prompts the question: what is the origin and structure of the small number of MR1 molecules that can be detected on the surface of most cells even in the absence of microbes [24,32,37]? One possible answer is that these molecules are in fact not empty but loaded with an as-yet-unidentified endogenous ligand that enables the egress of a small cohort of molecules from the ER. This does not appear to be the case, however, for the following reasons. In this scenario the hypothetical ligand would have to be produced in limiting and very small amounts because, as we describe below, MR1 expression is almost exclusively dependent on ligand availability and very few molecules reach the cell surface in the absence of VitBAg-producing microbes. However, when cells overexpress MR1 the number of molecules that reach the cell surface in the absence of VitBAgs increases proportionately with the level of protein synthesis, arguing against the existence of an expression-limiting endogenous ligand [24]. It is more likely that inside the ER there is an equilibrium between two conformational folding states of MR1 and that one, less abundant, can leave the ER without ligand, as has previously been described for MHC I molecules [19,40]. In cells overexpressing MR1, the abundance of the latter conformer would increase correspondingly. Further evidence for the escape of such empty molecules comes from experiments showing that MR1 surface expression increases in cells cultured at low temperature (26°C) [33], which would favor the stabilization and egress of empty molecules from the ER, again resembling MHC I [41,42]. The paradox is that empty MR1 molecules appear to be fully folded and are not less stable at the plasma membrane than those that have acquired ligands [24], unlike their MHC I counterparts. It is possible that while the physico-chemical conditions of the ER destabilize empty MR1 molecules, those on the cell surface are more favorable. Regardless, the functional significance of empty MR1 molecules on the plasma membrane is that, small as their number may be, they may be able to bind extracellular ligands on the plasma membrane. This may be important for the presentation of Ags that cannot reach the ER or endosomes in sufficient concentration to generate complexes with MR1 in these compartments, as described in the sections below. Another function for MR1 molecules expressed on the cell surface in the absence of microbially derived ligands is to promote MAIT cell selection in the thymus [43]. Whether the molecules involved in this function are associated with a self-ligand or correspond to the 'empty' conformers described above remains an open question.

### MR1 Acquisition of VitBAgs in the ER: Surface Expression and Presentation On Demand

At the time of discovery of VitBAgs, it was generally accepted that MR1 would load Ags in endosomal compartments based on the assumption that microbial Ags would largely be acquired from the extracellular environment by endocytosis, or would be released to the lumen of phagosomes harboring intracellular bacteria. The predominant hypothesis was therefore that MR1 followed an MHC II-like presentation pathway, loading Ags in endosomal compartments.

However, when we tested this hypothesis employing synthetic and pathogen-derived VitBAg ligands the data suggested a very different trafficking scheme.

We confirmed previous studies [9,10,33] showing that in the absence of ligand the majority of MR1 resides in the ER, both in primary cells and when MR1 is overexpressed [24]. This was confirmed by showing that under these conditions MR1 is sensitive to endoglycosidase H, and a hybrid MR1-GFP fusion protein colocalizes with the ER. More intriguingly, we also found that the ER is the location where MR1 loads VitBAgs [24]. The first hint that this was the case was provided using brefeldin A (BFA), showing that this inhibitor of ER egress blocked cell surface expression of MR1–VitBAg complexes in all cell types tested and largely inhibited MAIT cell activation by *Salmonella*-infected primary APCs. By contrast, inhibition of protein synthesis had little effect, highlighting that a pool of preformed MR1 molecules exists in the ER available for VitBAg capture [24]. These ER-resident MR1 molecules were maintained in an 'open' conformation, since they were not recognized by the monoclonal antibody 8F2.F9, which recognizes the fully folded form of MR1 [32]. We concluded that in the steady state without Ag, MR1 presentation is in the 'off' state (Figure 2). This sets MR1 apart from other human Ag-presenting molecules, which constitutively bind endogenous ligands or chaperones in the ER and traffick to the cell surface or to endosomes [44]. Most MR1 remains inside the ER as an immature molecule ready to capture its unstable metabolite ligands.

What happens when cells encounter VitBAgs in the extracellular medium? We observed that addition of VitBAgs to cells in culture caused MR1 to complete its folding into the 'closed' state inside the ER. VitBAg-dependent folding occurred even in the presence of BFA, confirming that MR1–VitBAg complexes are generated within the ER. The complexes then trafficked through the secretory pathway to the cell surface [24]. In support of this model, an siRNA screen for molecules involved in MR1 trafficking by Harriff *et al*. [31] found that Stx18, VAMP4, and Rab6,

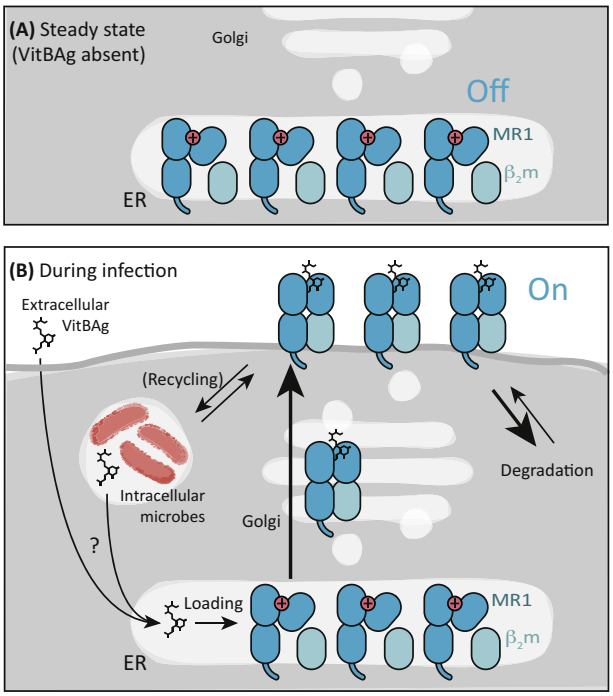


Figure 2. The Intracellular Pathway of MHC Class I (MHC I)-Related Protein 1 (MR1) Metabolite Presentation. (A) In the steady state and the absence of vitamin B antigens (VitBAgs), MR1 predominantly resides as an immature protein in the endoplasmic reticulum (ER) in an unfolded conformation, where the MR1 activation signal remains 'OFF'. (B) VitBAgs derived from the extracellular medium or intracellular microbes enter the ER via an unknown mechanism and load onto immature MR1, where the charged lysine-43 (K43) residue (+) is neutralized, triggering a conformational change that includes a strong $\beta_2$-microglobulin ($\beta_2$m) association. These complexes traffick through the Golgi apparatus to the cell surface, switching MR1 presentation 'ON'. MR1–VitBAg complexes remain at the cell surface for several hours. The molecules are subsequently internalized and delivered to the endocytic route for degradation, although a small number of molecules can recycle and return to the surface, potentially after acquiring new ligands in endosomes.

whose main role is to regulate protein trafficking between the ER and the Golgi [45–47], are involved in MR1 presentation of synthetic or *M. tuberculosis*-derived Ags.

Strikingly, the shift in MR1 structure and trafficking caused by ligand encounter relies on a chemical switch involving the K43 residue in the MR1 ligand-binding site rather than on simply ligand binding as is the case for MHC I. When MR1 binds a VitBAg, the positively charged amino group of the K43 residue forms a Schiff base with the carbonyl group of the VitBAg. This covalent association stably neutralizes the positive charge in K43. If K43 is mutated to alanine, which lacks the charged side chain, the mutant molecules (K43A) spontaneously fold and egress to the cell surface even without binding VitBAgs. Conversely, if the K43 residue is mutated to Arg (K43R) the mutant side chain now contains a positive charge that cannot be neutralized by a VitBAg because the Arg cannot form a Schiff base with the ligand [22], and these mutant MR1 molecules cannot escape the ER even when VitBAgs are present. The positively charged side chain of K43 thus functions as a 'destabilizing' motif that inhibits complete folding of MR1 (Figure 3). On binding a VitBAg, the K43 charge is neutralized via Schiff-base bond formation, triggering complete folding of the molecule and turning the MR1 presentation pathway 'on' (Figure 3). MR1 presentation is thus controlled by a molecular switch comprising a pathogen-driven post-translational modification of the Ag-presenting molecule.

The inevitable exception to the above rule appears to be a group of MAIT agonists that bind MR1 by making contacts with multiple residues in the cleft but without forming a Schiff base

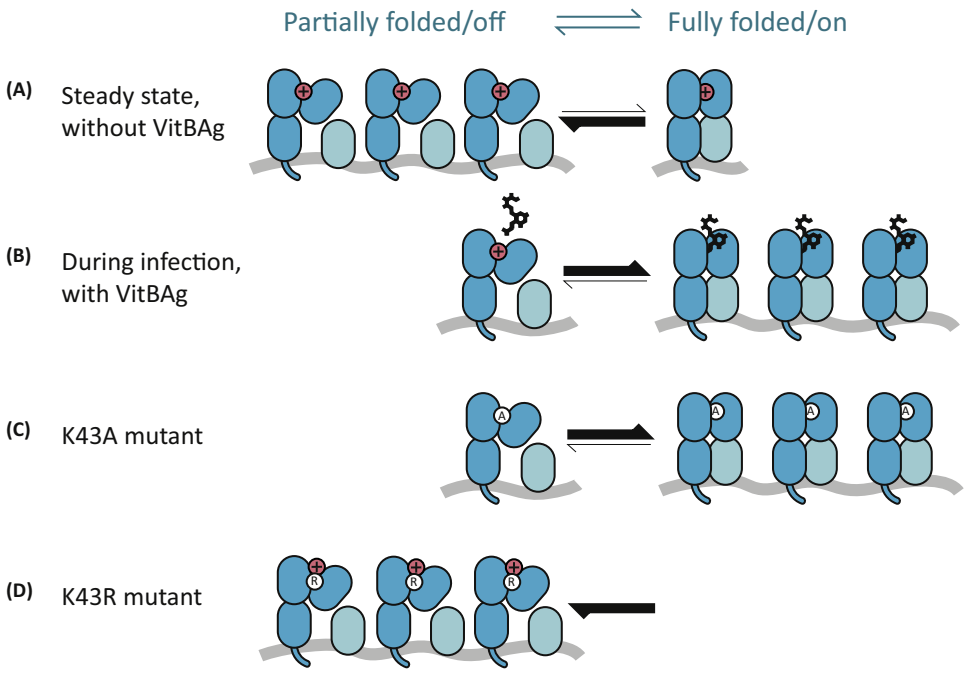


Figure 3. The MHC Class I (MHC I)-Related Protein 1 (MR1) Molecular Switch and Folding Equilibrium. MR1 exists in a conformational equilibrium between 'partially folded' (left-hand side) and 'fully folded' (right-hand side) that is dependent on a destabilizing lysine-43 (K43) residue. (A) In the steady state and the absence of vitamin B antigens (VitBAgs), the K43 side chain (red circle) remains charged (+) and inhibits the complete folding of MR1, and the equilibrium strongly favors the partially folded form, although some molecules may spontaneously fold and traffick to the cell surface. (B) During infection, the VitBAg (black) binds to and neutralizes the K43 side chain forcing the equilibrium to favor the fully folded conformation. (C) When K43 is mutated to alanine (K43A) there is no charged side chain in this position and the equilibrium shifts in favor of the folded form. (D) When K43 is mutated to arginine (K43R) the side chain remains charged and folding does not occur.

with K43: the ribityl lumazines [26] and diclofenac and its metabolite 5-hydroxy diclofenac [30]. However, these compounds induce little or no MR1 surface expression and are many fold less potent at activating MAIT cells than is 5-OP-RU [30]. This further supports the notion that effective presentation of MR1 Ags does not rely only on the association of the ligand with the binding site of MR1, as is the case for MHC I or II; it also requires the 'molecular switch' triggered by Schiff base formation. It is possible that the weak activation of MAIT cells induced by compounds that do not trigger this switch bind to the empty cohort of surface MR1 molecules described above, or in endosomal compartments as we discuss below.

The model that emerges from the experimental evidence gathered by different groups suggests that MR1 surface expression and Ag presentation are almost exclusively dependent on the acquisition of exogenous ligands within the ER. These ligands are by definition compounds that are not synthesized by the APC itself. Such ligands are synthesized by microbes and released to the extracellular environment, to the lumen of phagosomes harboring endocytosed bacteria, or to the cytosol of cells infected by intracytosolic bacteria. How these ligands access the lumen of the ER remains a mystery, but their trafficking route probably includes passage through endosomal compartments. This hypothesis is supported by studies showing that MR1 presentation to MAIT cells is much more potent after the uptake of bacteria into acidified compartments [18], as confirmed more recently by Ussher *et al*. [37]. Whereas these observations may be interpreted as evidence of endosomal loading, a more likely explanation is that the phagocytosed bacteria provide a more concentrated source of VitBAg in compartments from where the ligands can readily traffic to the ER. Further, lysis of bacteria in phagosomes probably releases VitBAg that could be efficiently transferred to the ER for MR1 binding. Validation of these scenarios awaits additional experiments.

### Does MR1 Acquire Ligands in Endosomal Compartments?

After arriving at the cell surface, MR1–VitBAg complexes are expressed for several hours before they are internalized and degraded, with a minor component recycling back to the surface [24] (Figure 2). MR1 molecules bound to an Ag acquired in the ER (e.g., 6-FP) could replace this ligand with a different ligand (e.g., 5-OP-RU) during trafficking through the recycling pathway (Figure 2) [24], providing a mechanism by which MR1 could acquire VitBAg in endosomal compartments. This might explain the results of other studies that reported endosomal MR1 loading, a pathway that may be dominant for some ligands derived from intracellular bacteria [17,31,37]. This is unsurprising because MHC I molecules, whose main site for Ag sampling is unquestionably the ER, can also exchange ligands in endosomes on recycling from the cell surface and thus present peptides produced in endosomes [48,49]. Further studies will be required to draw definitive conclusions about the relative contributions of the *de novo* (ER-based) versus the recycling (endosomal) routes of MR1 loading for MAIT cell activation. The recycling pathway relies on the accumulation of surface MR1–ligand complexes generated in the ER, so for Ag that can access only the ER, or both the ER and endosomes, the ER loading route is probably predominant. The intriguing possibility that remains is that certain ligands may never reach the ER and can be presented only via the recycling pathway.

### MR1 Endocytosis and Degradation: Terminating the MAIT-Activating Signal

The half-life of surface MR1 bound to VitBAg ligands of variable affinity, or not bound to any ligand at all, is relatively constant. This finding was unexpected because the half-life of MHC I molecules on the plasma membrane is strongly influenced by the affinity of their peptide ligands [50]. Therefore, prolonged presentation of MR1 Ag does not depend on the formation of stable complexes retained on the surface but on the sustained delivery of new MR1–ligand complexes generated in, and coming out of, the ER. As pathogens are cleared and the amount of available VitBAg metabolites wanes, the expression of MR1–VitBAg complexes decreases and the MR1 pathway returns to the off state. As a corollary, MR1 presentation is tightly regulated by the

availability of Ags and can provide information in real time to MAIT cells on the presence of VitBAg-producing pathogens.

## Concluding Remarks and Future Directions

The discovery that MR1 presents the broadly conserved family of VitBAgs has expanded the types of Ag that can be recognized by T cells. It has also allowed dissection of the intracellular events that characterize this novel Ag presentation pathway and opened an exciting new field with several important questions (see Outstanding Questions). There are discrepancies in the literature regarding the compartments where MR1 predominantly acquires its ligands, but this will probably be clarified with the analysis of more types of synthetic and microbe-derived ligands and the application of new analytical tools.

One major question is the identity of the putative components of the machinery required for efficient production of MR1–VitBAg complexes. It is likely that MR1 requires chaperones to stay in an open, Ag-receptive state in the ER and additional molecules may be involved in the step of VitBAg loading itself. Once MR1 leaves the ER and reaches the plasma membrane, additional sorting and trafficking machinery must be involved in the regulation of its recycling versus endosomal degradation. Characterization of these accessory molecules will illuminate MR1 biology and offer targets for the inhibition or enhancement of VitBAg presentation to MAIT cells.

An area of ongoing work suggests that innate immune signaling may also affect MR1-mediated MAIT cell activation [37,51]. Ussher *et al*. [37] recently provided compelling evidence in support of this notion; for example, showing that an inhibitor of NFκB signaling abolished MR1-dependent MAIT cell activation. A question that remains is whether innate signals regulate MR1 presentation *per se* or, rather, the recognition of the Ag and subsequent activation of MAIT cells through other mechanisms, such as the expression of costimulatory receptors or the release of activating cytokines.

A more intriguing question pertains to the discovery that MR1 loads exogenous VitBAgs in the ER: how do these metabolites access this compartment? Are there transporters dedicated to importing VitBAgs from the extracellular space or the lumen of endosomes to the cytosol and from there to the ER? Or do the metabolites follow a retrograde vesicular transport pathway that does not require their transit through the cytosol? Defining this novel pathway of Ag handling and presentation is critical to understanding the MR1–MAIT cell system, and its molecular components again represent potential targets for immunomodulation.

### Outstanding Questions

What is the cellular machinery required as MR1 performs its unique task of metabolite presentation? Other Ag-presenting molecules utilize dedicated or more general chaperones to capture and present their Ags. It is likely that chaperones will be involved in processes such as metabolite loading onto ER-resident molecules, maintaining MR1 in a partially folded state in the ER, and trafficking of MR1–Ag complexes to and from the plasma membrane.

Recent work suggests that innate immune signals may regulate metabolite presentation by MR1; however, little is known about the mechanism or the extent to which this impacts the MR1–MAIT cell axis.

Continuing from the discovery that MR1 loads VitBAgs in the ER, what is the mechanism by which the VitBAg metabolites access this compartment? MHC I primarily relies on TAP to import peptides into the ER from the cytosol. Are similar specific transporters used to traffic VitBAgs from outside the cell into the cytosol and then into the ER? Or do the antigens remain inside endosomes before being deposited into the ER via retrograde transport?

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