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MR1 Antigen Presentation to MAIT and other MR1-Restricted T Cells

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Abstract:

MHC antigen presentation plays a fundamental role in adaptive and semi-invariant T cell immunity. Distinct MHC molecules bind antigens that differ in chemical structure, origin and location and present them to specialized T cells. MHC class I-Related Protein 1 (MR1) presents a range of small molecule antigens to MR1-restricted (MR1T) lymphocytes. The best studied MR1 ligands are derived from microbial metabolism and are recognized by a major class of MR1T cells known as Mucosal Associated Invariant T (MAIT) cells. Here, we describe the MR1 antigen presentation pathway: the known types of antigens presented by MR1, the location where MR1-antigen complexes form, the route followed by the complexes to the cell surface, the mechanisms involved in termination of MR1 antigen presentation and the accessory cellular proteins that comprise the MR1 antigen presentation machinery. The current roadmap of the MR1 antigen presentation pathway reveals potential strategies for therapeutic manipulation of MR1T cell function and provides a foundation for further studies that will lead to a deeper understanding of MR1-mediated immunity.

Introduction:

Classical MHC class I (MHC-I) and MHC class II (MHC-II) molecules bind a large variety of peptides derived from the cytosolic and endosomal degradation of proteins, respectively, and present these on the surface of antigen presenting cells[1]. The classical MHC system of detection of threats to homeostasis provides the highest level of specificity, inter-cellular cooperation and cellular specialization in the immune system. In contrast, the cell-autonomous innate immune system allows for the detection of common pathogen components via pattern recognition receptors, which are expressed by most cells[2]. Non-classical MHC presentation sits in between these extremes on the spectrum between specificity versus frequency: it is based on the recognition of a limited variety of molecules by a relatively abundant type of T lymphocyte. For example, common lipids are presented to semi-invariant NKT and CD1-restricted T cells by members of the CD1 family of non-classical MHC molecules[3,4]. The most highly conserved, but arguably least-understood non-classical antigen-presenting molecule is MHC class I-Related protein 1 (MR1), which is expressed at low levels by diverse cell types [5-8]. The T lymphocytes that recognize MR1-presented antigens are known as MR1-restricted T (MR1T) cells.

The first antigens that were unequivocally identified as MR1 ligands consist of modified metabolites of the biosynthesis pathway of Vitamin B2 (riboflavin)[13,14]. These ligands are here collectively referred to as *Vitamin B-related Antigens* (VitBAgs). As riboflavin is synthesized by yeast and most bacteria[15] but not by mammals, VitBAgs provide a molecular signature for these microbes[13]. The MR1-VitBAg complexes are recognized by a subset of MR1T cells termed Mucosal Associated Invariant T (MAIT) cells[14,16-19]. These cells express a distinct TCR and follow a different developmental pathway compared to other T lymphocytes (**Box 1**)[13,14,16,17,20-25]. The development of MAIT cells in the thymus[17,25,28] and their recruitment, expansion, and TCR-mediated activation is strictly dependent on MR1-VitBAg presentation[29,30] (**Fig. 1**). MAIT cells comprise the majority of MR1T cells, are abundant (1-10% of all T cells in the blood)[31,32] and have been implicated in immunity to bacterial infection[5,33-35], wound healing[30,36,37], and regulation of the microbiome[38,39].

There are two additional subgroups of MR1T cells that share some, but not all features of MAIT cells[9-12]. Here we refer to these as "*non-canonical MAIT*" and "*atypical MR1T*" cells (see **Box 1**). The development and function played by these two types of MR1T cells have not been as extensively characterized as they have been for MAIT cells. They secrete cytokines upon

recognition of MR1-ligand complexes on tumour cells[40-42] and can display cytotoxic activity against a variety of cancer cells, indicating they may be specialized in anti-tumour immunity (**Fig. 1**). However, the ligands recognized by non-canonical MAIT and atypical MR1T cells remain unknown.

The monomorphic nature of MR1 (**Box 2**) and the roles played by MR1T cells in immune stimulation implies that this recognition system could potentially be harnessed as a pan-human antigen-specific immunotherapy against riboflavin-producing pathogens or cancer[40]. The characterization of the full range of functions played by all three types of MR1T cells, and of the ligands they recognize, is therefore a major driver of research in the field of MR1 biology.

Another central question in MR1 research is: how do MR1 molecules present their ligands? Every antigen presentation pathway is defined by the origin and chemical composition of the antigen, the structure of the MHC(-like) molecule that presents it, and the site where the complexes form. For example, the presentation of cytosolically- and endosomally-generated peptides by MHC-I and MHC-II requires each molecule to follow a distinct intracellular trafficking pathway[43,44]. In turn, each pathway involves a unique set of accessory molecules. The components of this machinery are potential targets for enhancement or disablement of T cell antigen recognition by drugs[45] or pathogens[46]. Characterization of the location, processes and components of the machinery involved in MR1 antigen presentation will lead to a better understanding of the function of MR1T cells and the development of new therapies.

In this Review, we first describe the nature of MR1 ligands, their origin and recognition by MR1T cells. We follow with a detailed description of the MR1 antigen presentation pathway, from MR1 synthesis in the ER through formation and display of MR1-ligand complexes on the cell surface to MR1 degradation in the endosomal route. We indicate the areas most in need of additional study and suggest research directions that may lead to therapeutic applications of MR1T cells.

[H1] The nature of MR1 ligands

The description of VitBAgs as MR1 ligands that are recognized by MAIT cells was a turning point for the field. The riboflavin biosynthesis pathway produces the intermediate 5-amino-6-D-ribitylaminouracil (5-A-RU)[14], a highly labile compound that can combine with glyoxal or methylglyoxal, two ubiquitous metabolites, to form single-ring pyrimidines (**Fig. 2** and **Table**

1). The best studied of these pyrimidine VitBAgs is 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)[14] (**Fig. 2** and **Table 1**). Alternatively, 5-A-RU or 5-OP-RU can give rise to dual-ring ribityl lumazines (**Fig. 2** and **Table 1**). Both the pyrimidines and ribityl lumazines can bind to MR1, but the pyrimidines, and in particular 5-OP-RU, are orders of magnitude more potent at MAIT cell stimulation than the lumazines[26,47]. The lack of potency of the lumazines is primarily due to their inability to bind covalently and induce the conformational changes required for MR1 surface expression, as described in more detail below.

The ability of particular bacterial species to stimulate MAIT cells via MR1 strictly correlates with their ability to synthesize riboflavin[48]. Since riboflavin is produced by microbes and not mammals, the resulting VitBAgs can be deemed pathogen associated molecular patterns (PAMPs). Indeed, VitBAgs are exceptionally conserved and prevalent; the majority of bacteria[49] and many fungi contain the genes required for riboflavin synthesis. VitBAgs are most abundant when microbes are actively multiplying and producing riboflavin in the process[50]. As VitBAgs are also extremely labile and unstable[47], the detection of MR1-VitBAg complexes by MAIT cells is a sign of actively replicating microbes. In mice, it was shown that the presentation of VitBAgs by MR1 is necessary and sufficient for MAIT cell selection in the thymus[17,29], stimulation of MAIT cells in the periphery[29,30] and MAIT-mediated immunity against pathogens that produce vitamin B[33,35,51]. Moreover, a patient suffering from recurring viral and bacterial skin infections was found to express a mutant MR1 molecule that cannot present 5-OP-RU. This individual presented with a severely reduced MAIT cell compartment[52], confirming a conserved role for VitBAg presentation across species (**Box 2**). The effects of this mutation indicate that MAIT cells are critical for host defense at barrier surfaces, with the caveat that the patient also has expanded numbers of γδ T cells and carries an additional mutation in the *IFIH1* gene, which is involved in virus RNA detection, alterations that may also contribute to disease susceptibility[52]. In summary, the role and physiological relevance of VitBAgs in the MR1-MAIT cell axis, although incompletely understood, is incontrovertible.

[H2] MR1 ligands beyond VitBAgs

MR1 ligands that are capable of eliciting effective, MR1T cell-mediated responses against pathogens or tumors could potentially be attractive as therapeutics. Given that MR1 is monomorphic (**Box 2**), strategies that activate the MR1T cell compartment have a major

advantage over those that employ conventional $CD4^+$ and/or $CD8^+$ T cells where MHC polymorphism requires matching therapies to individuals that express the right MHC allotype(s)[53]. Structural studies have revealed the MR1 antigen binding cleft has enough flexibility to accommodate a wide variety of molecules[7,54,55]. The therapeutic potential of MR1T cells is driving a vigorous search for MR1 ligands beyond VitBAg, as reviewed recently[56] and summarized below and in **Table 1**.

The non-VitBAg MR1 ligands described so far can be grouped into two categories (**Fig. 2**): (i) non-microbial folate derivatives; and (ii) synthetic drugs derived from natural scaffolds or identified *in silico* as potential MR1 binders. Examples of the first group are the Vitamin B9-derived pterins such as 6-FP[14] and Ac-6-FP[57] (**Table 1**). These ligands are efficiently presented by MR1 but do not elicit MAIT cell activation. However, they can compete with VitBAg for MR1 binding and thereby inhibit MAIT cell responses, so can function as immunosuppressive drugs in vivo as shown in mouse models[27,58]. On the other hand, they can stimulate some rare non-canonical MAIT cells[11], so they may play a role in immunity, but this is speculative at present. Other studies have also described or provided evidence for microbial MR1 ligands that are distinct from VitBAgs, but their contribution to immunity against infection has not been established yet[59,60] (**Table 1**). It should be noted that the expression of CD8, an MHC-I and MR1 co-receptor, enhances antigen recognition by some MR1T cells[61]. High CD8 expression may enable MR1T cells that express TCRs of low affinity to recognize these ligands.

The definition of the structural features that enable known ligands to fit into the MR1 antigen-binding cleft has enabled informed predictions of new natural or synthetic ligands. Using this approach, a metabolite of diclofenac (5'-hydroxy diclofenac), a common non-steroid anti-inflammatory drug, was identified as a ligand of MR1[27] (**Table 1**). Interestingly 5'-hydroxy diclofenac can activate some MAIT TCRs and can also synergize with 5-OP-RU to increase MAIT cell activation when VitBAgs are present at low concentration in vitro[27]. Using an alternative approach, synthetic analogs of known ligands of MR1 have led to the synthesis of new compounds with MAIT cell immunomodulatory properties[26,47,62,63], these include stable analogs of 5-OP-RU (JYM72[47]) and 5-A-RU (a prodrug of 5-A-RU[64]) (**Table 1**).

Three studies have provided evidence for an additional category of MR1 ligands, in this case derived from mammalian cells. The first described atypical MR1T cells that recognize two 'families' of tumor cell-derived MR1 ligands that were out-competed by the *bona fide* MR1

ligand, 6-FP[41] (**Table 1**). The second study provided evidence for atypical MR1T cells that recognize metabolites produced by different types of tumors but not healthy cells[40] (**Table 1**). A follow-up study validated these conclusions and demonstrated that recognition of some of the metabolites did not require expression of the Lys43 residue in MR1, which is required for VitBAg presentation (see below)[42]. The identity of the antigen recognized by the atypical MR1T cells reported in these studies remains undescribed.

More work is required to demonstrate the physiological role of the putative ligands recognized by non-canonical MAIT and atypical MR1T cells, whether pathogen- or host-derived, subjecting them to the same standard of proof *in vitro* and *in vivo* that was applied to VitBAg recognition by MAIT cells. Nevertheless, even if these ligands do not elicit physiological immune responses, they may be useful to recruit the MR1T cells that recognize them for therapeutic applications

[H2] The location of MR1 ligand formation and release

The identification of the site where MR1 ligands are generated is of interest because it can help the characterization of the mechanism of MR1 antigen presentation and predict the participation of accessory molecules, as it did for the MHC-I and MHC-II pathways[43,44]. Where are MR1 ligands produced?

Intracellular pathogens such as *Mycobacterium tuberculosis*[65], *Salmonella enterica* serovar Typhimurium[7,66], and *Shigella flexneri*[34,67], which multiply inside endosomes or in the cytosol, produced VitBAgs. MR1 molecules bind these intracellular ligands and present them on the surface of the infected cells, which activates MAIT cells that then kill the cells and/or secrete inflammatory cytokines[6,34]. Other MR1 ligands are presented following their capture by MR1-expressing cells, as described above for VitBAgs released by commensal microbes to the extracellular milieu[29,30]. Germ-free mice lack a microbial source for extracellular VitBAgs and do not generate MAIT cells[17]. This defect can be rescued by microbial recolonization, or more simply by applying 5-OP-RU in barrier tissues such as on the skin or in the gastrointestinal tract. Remarkably, the injected VitBAgs can reach the thymus and enable the positive selection of MAIT cells[29]. MR1 presentation of extracellular VitBAgs at barrier tissues such as the skin[30] enables MAIT cells to set up residence at sites that are constantly exposed to the microbiota, likely protecting against infection by microbial pathogens.

The origin of non-microbial MR1 antigens is unclear, as is their identity[40-42]. These antigens are most likely endogenous, i.e. produced by the antigen presenting cell itself, but it is also possible that they are secreted by other cells and captured from the extracellular milieu by the MR1-expressing cells. These are important questions given that atypical MR1T cells have been implicated in immunity against cancer[40-42,68]. Here, the targeted delivery of tumor-specific MR1 ligands might further stimulate these cells for therapeutic purposes. Conversely, autoimmune responses mediated by self-MR1 ligands[10] might be curtailed by preventing the capture or presentation of the ligand.

Studies undertaken to address the nature, origin and mechanisms of MR1 ligand capture dovetail with advances on the characterization of the site where MR1 binds the ligands, the machinery involved in formation of the MR1-ligand complexes, and the intracellular pathway followed by MR1 molecules from synthesis to degradation.

[H1] The MR1 Antigen Presentation Pathway

At steady state and in the absence of infection, MR1 is barely detectable on the surface of most human or mouse cells. However, it is readily up-regulated in cells exposed to 5-OP-RU and other ligands (**Fig. 3 and 4**)[7,13,14,57,69]. This mode of antigen display can be described as 'presentation on demand' and it sets MR1 apart from other antigen-presenting molecules such as MHC-I and -II, which are constitutively expressed on the plasma membrane bound to self ligands[1]. The distinct behavior of MR1 suggests that its surface expression is tightly controlled, thereby preventing inappropriate MR1T cell activation in the absence of infection. Notably, the TCR of some γδT cells can interact with a region of MR1 that does not include the antigen-binding site[70,71]. Low MR1 expression in the absence of infection may prevent potentially deleterious stimulation of such γδT cells and perhaps other T lymphocytes as well. However, such antigen-independent recognition of MR1 could potentially be exploited therapeutically using natural or synthetic ligands that are capable of inducing MR1 expression. Conversely, potential autoimmunity mediated by MR1 presentation might be prevented with synthetic compounds that are capable of inhibiting MR1 delivery to the cell surface, as has been demonstrated for the synthetic small molecule DB28[72].

[H2] MR1 retention in the Endoplasmic Reticulum

The effect of ligands on MR1 surface expression might be mediated at the transcriptional, translational or post-translational level. Since inhibitors of protein synthesis do not prevent the

up-regulation of MR1 surface expression in the presence of ligands, it must be regulated by post-transcriptional mechanisms[7]. These might affect the rate of MR1 deposition on the cell surface, or the rate of turn-over at the cell surface, as is the case for MHC-II[73]. The reported association of MR1 with the MHC-II chaperones CD74 and H-2DM suggested that surface expression of MR1 may be regulated by these chaperones in a similar way to MHC-II[74], including transport to endosomes by CD74, but subsequent studies discarded this hypothesis. Microscopy analysis of cells that were not exposed to MR1 ligands showed the near absence of MR1 in any compartment other than the endoplasmic reticulum (ER) as opposed to MHC-II which is found in endosomal compartments or the cell surface[7,75-78]. Furthermore, the MR1 carbohydrate in these cells is sensitive to the glycosidase EndoH[7,72,75], an enzyme that can only remove the carbohydrate of glycoproteins that reside in the ER. It is now well established that MR1 is mostly retained in the ER in the steady-state and that it only traffics from the ER to the plasma membrane in cells that are exposed to MR1 ligands (**Fig. 3 and 4**)[7,75]. The few molecules found outside the ER may be bound to an unknown ligand or, more likely, may be devoid of any ligand.

[H2] The role of ER chaperones

MHC molecules with empty antigen-binding sites are inherently unstable and prone to form potentially toxic aggregates with themselves or other polypeptides[79-82], so it was expected that the pool of MR1 molecules retained in the ER would contain some ligand in its antigen-binding site. However, studies with conformational-sensitive monoclonal antibodies (mAbs) showed that the majority of MR1 retained in the ER is in a semi-folded 'empty' state[7,75] (**Fig. 3**). Two empty conformers co-exist, one free and the other bound to β2m, (the smaller protein subunit shared with MHC-I and CD1 molecules (**Table 2**)[75], and both are stabilized via association with ER chaperones.

To identify these chaperones and other components of the MR1 presentation machinery, two genome-wide screens were used to detect proteins required for MR1 expression upon ligand addition. These identified ATP13A1 (**Table 2**)[75,83], a protein that functions in mammalian cells as a translocase to remove misdirected mitochondrial proteins out of the ER[84]. Cells lacking ATP13A1 were defective at MR1 antigen presentation of both extracellular ligands (5-OP-RU) and antigen derived from intracellular bacteria because they contained a smaller pool of MR1 in the ER, though the underlying cause remains unknown (**Fig. 3**)[83]. One of the screens also revealed a role for the MHC-I peptide loading complex (PLC) components TAP1 and Tapasin[75]

(**Table 2**). Studies to investigate the role of the PLC in MR1 stabilization, which were carried out before the description of natural MR1 ligands, were inconclusive[5,17,78], but a more recent study showed that MR1 immunoprecipitation pulled-down all the components of the PLC including MHC-I[75]. Each PLC normally contains two MHC-I molecules[85], so it appears that at least one of these molecules can be replaced with MR1. The deletion of Tapasin in cell lines and primary cells impaired MR1 antigen presentation, but only partially because cells also express TAPBPR, a Tapasin homolog that does not bind to the PLC but also chaperones MHC-I[86,87] (**Table 2**). Both Tapasin and TAPBPR can chaperone MR1[75], but the MR1-Tapasin complexes can be found on their own or associated to the PLC[75] whereas the MR1-TAPBPR complexes never associates with the PLC[88,89].

What is the role of Tapasin and TAPBPR in MR1 antigen presentation? Both chaperones play a dual role in the MHC-I presentation pathway: they stabilize antigen-free molecules and also promote a cycle of binding and release of peptide ligands to the MHC-I antigen-binding site in a process termed *editing*[86,87,89-93]. Once a peptide of relatively high affinity binds, the MHC-I-peptide complexes dissociate from these chaperones, exit the ER and traffic to the cell surface[43]. However, Tapasin and TAPBPR do not appear to play an editing role in MR1 antigen presentation. The interaction of MR1 with TAPBPR widens the MR1 antigen-binding cleft and can increase both the loading and dissociation rates of the non-covalently bound ligand diclofenac[94]. On the other hand, the major structural changes seen in MHC-I upon peptide binding were not mirrored during MR1 metabolite loading[94], and the TAPBPR-MR1 interaction was not influenced by antigen binding[75,94], which argues against a 'metabolite editing' function. More importantly, the proportion of MR1 molecules that associate with ligands in cells incubated with VitBAg is not affected by the absence of the two chaperones[75]. The role of Tapasin and TAPBPR in physiological settings of bacterial infection remains to be established, but their function appears to be to stabilize empty MR1, allowing the maintenance of a pool of ligand-receptive molecules in the ER. This hypothesis is supported by the observation that cells lacking both chaperones have a sharp reduction in the size of the MR1 pool in the ER[75], which severely impairs MR1 presentation (**Fig. 4**). Given their pleiotropic roles, it is pertinent to ask whether the evolution of Tapasin and TAPBPR was primarily driven by their MHC-I stabilization and peptide-editing function, or by their role in the maintenance of an empty MR1 pool. MHC-I molecules are polymorphic and not all allomorphs require Tapasin/TAPBPR[88,95,96], suggesting the highly conserved structure of MR1 may have played a more dominant role than MHC-I in the evolution of the two chaperones.

Cells exposed to pathogen components up-regulate the production of new MR1 molecules that may contribute to antigen presentation[97,98], but the strong dependence of the pathway on the size of the ER pool at the time of antigen encounter[75] (**Fig. 4**) sets MR1 apart from other antigen-presentation pathways that rely primarily on newly-synthesized molecules[99]. As the half-life of VitBAg is limited[47], a reservoir of empty MR1 ensures that even small amounts of VitBAg can be captured, protected from degradation via MR1 binding, and displayed to MAIT cells on the cell surface within a short period of time (**Fig. 4**). Further evidence for the importance of the 'empty' MR1 pool comes from studies of viruses that specifically interfere with MR1 antigen presentation. Infection with several members of the herpesviridae family induces the delivery of MR1 to the ER-associated degradation pathway[100] and reduces the size of the empty MR1 pool[101-103]. Studies have identified several viral factors (immunoevasins) that target MR1: for example, US9 from human cytomegalovirus depletes the intracellular MR1 pool[103], while US3 from herpes simplex virus-1 and its homolog ORF66 from varicella zoster virus both downregulate surface MR1[101,102] (**Table 2**). Yet the deletion of each of these factors from their respective parental viruses does not completely prevent MR1 degradation, implying that there are other as-yet undefined immunoevasins that target MR1[101-104]. The virus may not benefit directly from the degradation of MR1 (i.e. by blocking MR1 presentation of viral antigen), but indirectly[104]. Viruses that cause barrier disruption, such as herpes viruses, may induce the recruitment of MAIT or other MR1T cells that recognize ligands released by commensal bacteria or stressed tissues. Such MR1T cells might contribute to establishing an inflammatory environment that is hostile to the virus. Inhibition of MR1 presentation through the reduction of the MR1 ER-resident pool would therefore reduce MR1T cell recruitment and benefit the virus. Though speculative at present, it is also possible that cells infected with viruses undergo metabolic changes that result in the production of new MR1 ligands, a situation analogous to the reported production of MR1T cell neo-antigens by cancer cells[40,41].

[H2] MR1 ligand binding in the ER

The identification of the intracellular location where MR1 binds its ligands has been the subject of intense and controversial investigation. As MR1 ligands are captured from the extracellular environment by endocytosis, or produced within the lumen of endosomes that harbor bacteria, the initial assumption was that antigen binding would take place in the endosomal route, as this is where both MHC-II and CD1 molecules bind endocytosed ligands[3,43,99]. However, MHC-II and CD1 constitutively migrate to endosomes, whereas MR1 molecules are mostly retained in

the ER. This paradox was resolved with the discovery that MR1 primarily binds extracellular ligands in the ER[7]. Multiple experimental approaches support this conclusion, the most revealing of which is arguably the use of the synthetic 5-OP-RU derivative, *MR1 antigen Analog-tetramethylrhodamine* (MAgA-TAMRA)[75]. The fluorescent TAMRA motif on this functionalized ligand enables the measurement of its uptake and localization within cells and doubles as an epitope tag for the localization, pulldown, and detection of MR1-antigen complexes with anti-TAMRA mAb[75]. This allowed the identification of the ER as the site of MR1-ligand complex formation[75], confirming earlier indirect evidence obtained with analysis of 5-OP-RU binding[7] (**Fig. 3**). Moreover, DB28 was found to inhibit MR1 presentation by binding to MR1 in the ER, where it causes "entrapment" of the complexes within the compartment rather than egress to the cell surface[72]. Although the underlying mechanism of retention is not completely understood[72], the effect of DB28 complements the observations made with MAgA-TAMRA and other VitBAg ligands in defining the ER as the primary site of MR1 ligand acquisition[75]. The observation that some MR1 ligands are recognized by atypical MR1T cells without inducing detectable changes in surface MR1 expression has been interpreted as evidence of ligand binding to MR1 molecules already expressed on the cell surface[40,41]. However, T cells are extremely sensitive to very small numbers of MHC-ligand complexes[105,106] and it is also possible that such ligands did bind to a small number of ER-resident MR1 molecules, sufficient to cause MR1T cell activation but not enough to increase the overall levels of MR1 on the cell surface above the limit of detection. Indeed, small amounts of VitBAgs bind to ER-resident MR1 and can activate MAIT cells without causing apparent changes in surface MR1 expression[7]. We conclude that although ligand acquisition outside the ER remains a possibility (see below), the predominant site for assembly of MR1-antigen complexes is the ER.

[H2] Release of MR1 from the ER

When VitBAgs reach the ER they bind to MR1, triggering a conformational change that releases the resulting complex from chaperone binding and enables its transport to the cell surface[75] (Fig. 2). This is analogous to the release of ER-resident MHC-I molecules from the PLC upon binding of peptide antigens that are transported by TAP[43]. However, occupancy of the antigen-binding site is not sufficient to trigger MR1Transport to the cell surface[13,26,27,31,59]. Ribityl lumazine antigens can readily bind to the MR1 cleft[14] but do not readily recruit MR1 to the cell surface, and, compared to 5-OP-RU, are at least 4 orders of magnitude less potent at activating MAIT cells (**Table 1**)[26,27]. The reason for this paradox is that the change in MR1

conformation that is required for ER egress is driven by a mechanism that is unique to MR1 presentation: the formation a covalent bond (a *Schiff base*) between the antigen and a conserved lysine present in the MR1 binding site (K43, **Fig. 3C**)[13]. The formation of this bond neutralizes the positive charge of K43. Interestingly, if K43 is mutated to alanine (K43A), the now-neutral side chain allows surface expression of the mutant $MR1^{K43A}$ molecule, even in the absence of ligands. Conversely, if K43 is mutated to arginine ($MR1^{K43R}$), a residue that is also positively charged but cannot be neutralized by Schiff base bonding with VitBAgs, the molecule never leaves the ER[7]. This implies that MR1 release out of the ER is not caused by ligand occupancy *per se*, but by the neutralization of K43 via covalent ligand binding. It is likely that the ribityl lumazines can associate with MR1 but do not trigger ER egress because they do not establish this covalent bond[7,13,14,57,69].

It is not entirely clear how the unoccupied K43 side chain mediates ER retention, but binding of Schiff base-forming ligands has been shown to induce conformational changes in MR1[7,75]. Quality control chaperones monitor protein folding in the ER and prevent incompletely folded molecules from exiting this compartment[107]. Therefore it is hypothesized that the K43 side chain acts as a destabilizing motif that prevents complete MR1 folding. The semi-folded molecules bind to Tapasin or TAPBPR and are retained in the ER until MR1 binds a Schiff base-forming ligand that causes complete folding, detachment from the two chaperones and eggres to the cell surface[112]. The structure of incompletely folded MR1 has not been determined yet, but would likely provide insight into how the K43 side chain controls MR1 conformation.

The role of Schiff base-bonding in MR1 function was illustrated with the discovery of a human MR1 mutant molecule where the Arg9 residue is changed to His (R9H mutation; **Box 2**). The $MR1^{R9H}$ molecule is unable to form a Schiff base with 5-OP-RU and a patient homozygous for the R9H mutation lacked MAIT cells[52]. The observation of this mutation and the conservation of K43 throughout evolution lead us to the conclusion that MR1 is adapted to present ligands capable of forming Schiff-base bonds. Exceptions exist and unidentified tumor antigens can be presented to atypical MR1T cells by wild-type and mutant $MR1^{K43A}$ molecules[41]. These ligands may induce the change in conformation required for MR1 egress out of the ER without forming a covalent bond, or they may bind to the few, probably empty molecules found outside the ER in the steady state.

[H2] MR1Trafficking to the Plasma Membrane

Following ligand binding, MR1-ligand complexes leave the ER, cross the Golgi apparatus and traffic to the plasma membrane[7,75]. The route followed is most likely the default secretory pathway. Alternatively, MR1 might traffic through endosomal compartments on the way to the surface, but MR1 lacks the sorting signals that are required to follow this pathway. It is also unlikely that a chaperone binds to and delivers MR1 to endosomes because no such protein has been revealed in pull-down experiments[75] or genetic screens[69,75,83]. An analysis of the role of 115 genes involved in the regulation of protein trafficking along the secretory pathway showed that proteins with known functions in transport to, along or out of the Golgi complex, such as VAMP4, RAB6 and STX16, participate in MR1 presentation of ligands produced by intracellular bacteria[69] (**Fig. 3** and **Table 2**). These findings also indicate that MR1-ligand complexes traffic to the plasma membrane via the default secretory pathway.

[H2] MR1 endocytosis, recycling, and lysosomal destruction

All plasma membrane proteins are endocytosed in clathrin-coated vesicles and other types of vesicles[113,114] that are generated throughout the plasma membrane[113,114]. Any surface protein that happens to be present in the portion of membrane that contributes to vesicle formation is endocytosed passively. This is the mechanism of endocytoisis followed by MHC-I molecules[43]. In contrast, other membrane proteins such as CD1d are actively recruited to sites of vesicle formation because they contain cytosolic motifs that are recognized by the endocytic machinery[3]. As a consequence, CD1d is endocytosed at a much higher rate than MHC-I. MR1-ligand complexes are endocytosed at an intermediate rate (half-life of 2-4 hrs)[67].Replacement of the cytosolic tail of MR1 with the cytosolic tail of CD1d accelerated endocytosis[67], whereas addition of Green Fluorescent Protein (GFP) to the cytosolic C-terminus of MR1 reduced the rate of endocytosis[67]. This indicates that the MR1 tail contains an internalization motif that is less potent than that found in CD1d and is disabled by the addition of GFP. We identified this motif as the conserved residues 313-316 (YLPT) of human MR1[67]. It partially resembles the canonical YXXΦ sequence of residues recognized by AP2, a cytosolic adaptor complex that plays a central role in Clathrin-mediated endocytosis[115]. Furthermore, a genome-wide CRISPR/Cas-9 library screen of proteins involved in MR1 endocytosis identified AP2 as the most prominent hit[67]. An analysis of the effect of inhibitors of clathrin-mediated endocytosis and of ablation of AP2 components confirmed the role of AP2 in MR1 internalization[67]. In the evolutionarily conserved MR1 motif, residue Tyr313 plays a central role in AP2 binding, but the absence of a bulky hydrophobic residue (Thr) in position 316 reduces the affinity of the

interaction[67]. Therefore, MR1 contains a suboptimal AP2 recognition motif that makes the rate of MR1 endocytosis slow enough to enable detection of ligands by MR1T cells, but fast enough to terminate presentation shortly after the source of the ligand has been eliminated[67].

Endocytosed membrane proteins can recycle back to the plasma membrane or traffic to lysosomes, where they are degraded[113,114]. Approximately 95% of the MR1-antigen complexes that undergo endocytosis are degraded[7,67]. The remaining 5% are recycled after transit through early/recycling endosomes, where they can exchange their antigens with new ligands[7,64,69,116] (**Fig. 3**)[3,43,117]. Displacement of Schiff base-bound ligands from the MR1 antigen binding site may appear surprising, but in vitro assays found MR1-6-FP complexes generated in the ER and transported to the cell surface could exchange 6-FP for 5-OP-RU in endosomes[7,116]. This recycling pathway may enable the presentation of ligands that are endocytosed from the extracellular milieu, or are produced by bacteria within endosomes, but cannot reach the ER [69,116,118-120]. However, a caveat is that this pathway relies on the surface accumulation of MR1-ligand complexes that are generated in the ER, so its contribution to metabolite presentation under physiological conditions is unclear[121]. Impairing MR1 internalization did not prevent presentation of antigen endocytosed from the extracellular medium or produced by intracellular pathogens[67]. Furthermore, MR1-VitBAg complexes are unstable at pH<6 and dissociate from the β_2m subunit, so recycled molecules may not be able to bind ligands in compartments that are more acidic than early endosomes[67]. In conclusion, recycling does not appear to play a prominent role in MR1 antigen presentation, at least for the ligands that have been tested so far. It may be exploited for therapeutic purposes, though: a stable analog of 5-A-RU that contained a target sequence for the protease Cathepsin B[64] was cleaved in endosomes to produce an MR1 ligand that was presented by recycled molecules[72].

[H1] Concluding remarks and future directions.

The roadmap of the MR1 antigen presentation pathway is now reasonably well understood. A depot of ligand-free MR1 molecules that are stabilized by chaperones resides in the ER. Ligands that can reach the ER, fit into the antigen binding cleft and establish a Schiff base bond with MR1 residue K43 readily form covalent MR1-antigen complexes that traffic to the cell surface via the default secretory pathway. These complexes are endocytosed within hours and, although ~5% recycle back to the surface, potentially loaded with new ligands exchanged in endosomes, most are destroyed in lysosomes (Fig. 3). There are three areas that require further work and are likely to yield major advances in this field:

Firstly, we need a detailed description of the pathway, mechanisms and molecular participants in the transport of ligands for MR1 from the extracellular medium, from endosomes that harbor bacteria, or from the cytosol to the ER. Passive diffusion is an unlikely mechanism[75], but no specialized transporters of MR1 ligands (equivalent to TAP for MHC-I presentation[122]) have been described yet. Moreover, if transport across membranes is required, this may involve distinct transporters on the plasma membrane, endosomes and the ER.

An alternative mechanism for ligand transport that does not require transfer across membranes is via the lumen of vesicles that are involved in retrograde intracellular trafficking. Retrograde transport is a pathway by which bacterial toxins can reach the ER[123], and any protein can be passively transported to the ER via this pathway [124]. Whether the translocation of ligands for MR1T to the ER involves transporters or other means is unclear. However, the significance of the characterization of these mechanisms, and the potential therapeutic opportunities they may offer, cannot be overemphasized.

The second area that requires attention is to identify which cells, if any, dominate MR1 presentation in different immunological contexts. Insights into the cellular components that aid MR1 presentation may assist this search[34]. Cells that contain a larger pool of ER-resident MR1 are likely to present transient metabolites more efficiently than those with fewer molecules (**Fig 4**). In turn, the size of the MR1 pool may depend on the amount of Tapasin and TAPBPR made by the cell. TAPBPR is predominantly expressed by hematopoietic cells and its expression, like the expression of Tapasin, is induced by interferon-γ[88]. Professional antigen presenting cells (DC, macrophages and B cells) are obvious candidates to play a dominant role in MR1 antigen presentation, but this is still speculative and may vary with the type of immune challenge, i.e. pathogen infection, cancer or autoantigens.

Finally, the vigorous search for new MR1 ligands taking place at present may reveal new mediators of immune responses that may challenge the currently accepted views on the mechanisms of presentation, and on the cells involved, that apply to the ligands already known. Ligands made by the MR1 presenting cell itself, perhaps even within the ER, might have different requirements for presentation than those made by microbes. Synthetic versions of MR1 ligands may be used therapeutically, though these may require modifications of the natural structure to increase their stability [47,64] or enable them to reach the ER or other antigen-

505 loading compartments. For example, the 5-OP-RU analog JYM72 is stable and stimulates
506 MAIT cells *in vivo*, however it does not have the potency of the native ligand[47]. Further
507 modifications may improve the usefulness of synthetic MR1 ligands.
508
509 These are just some of the most prominent questions awaiting investigation in the field of MR1
510 antigen presentation. We anticipate quick and unexpected developments that will attract more
511 scientists to unravel the remaining mysteries of the interplay between MR1 and MAIT and
512 other MR1T cells. This knowledge may lead to new therapies against infection, cancer, allergy
513 and autoimmunity, and also to strategies that allow to manipulate non-immune functions such
514 as tissue repair and homeostasis[30,36,37,125].
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This study described MR1 conservation in mammalian evolution and found that MR1 and the MAIT TCRα chain (TRAV1) are intricately intertwined through evolution.

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983 **Table 1: Known and proposed MR1 ligands.**

Ligand family	Ligand	Abbreviation	Natural/ synthetic	Schiff base?	Effect on MR1 surface level[#]	Effect on MR1T cells*[#]	Effect *in vivo*?
Folate derivatives	6-formylpterin	6-FP	N	Yes[13]	Moderate upregulation[13]	MAIT^: inhibition[57] Non-canonical MAIT: activation (some)[11] Atypical MR1T: activation (some)[11]	Yes[58]
	Acetyl-6-formylpterin	Ac-6-FP	S	Yes	Potent upregulation[57]	MAIT: inhibition[57] Non-canonical MAIT: activation (some)[11] Atypical MR1T: activation (some)[11]	Yes[27, 58,66]
	2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal	Cp-C	S	Unlikely[126]	Potent upregulation[126]	MAIT: inhibition[126]	NT
Pyrimidines	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil	5-OP-RU	N	Yes[14]	Potent upregulation[14, 47,126]	MAIT: potent activation[14,26] Non-canonical MAIT: activation (some)[11] Atypical MR1T: activation (some)[11]	Yes[66]
	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil	5-OE-RU	N	Yes[14]	Potent upregulation[47, 126]	MAIT: potent activation[14]	NT
Ribityl lumazines	7-methyl-8-D-ribityllumazine	RL-7-Me	N	No[26]	Weak/no upregulation[27]	MAIT: weak activation[27,47]	NT
	6,7-dimethyl-8-D-ribityllumazine	RL-6,7-diMe	N	No[47]	NT	MAIT: weak activation[27,127] Non-canonical MAIT: activation (some)[60] Atypical MR1T: activation (some)[59]	NT
	7-hydroxy-6-methyl-8-D-ribityllumazine	RL-6-Me-7-OH	N	No[47]	NT	MAIT: weak activation[27] Non-canonical MAIT: activation (some)[60] Atypical MR1T: activation (some)[59]	NT

	Photolumazine I	PLI	N	No[59]	NT	MAIT: activation[59] Atypical MR1T: activation (some)[59]	NT
	Photolumazine III	PLIII	N	No[59]	NT	MAIT: activation[59] Atypical MR1T: activation (some)[59]	NT
Drugs and other synthetic ligands	3-formylsalicylic acid	3-F-SA	S	Yes[27]	Moderate upregulation[27]	MAIT: inhibition[27]	Yes[27]
	5-hydroxy-diclofenac	5-OH-DCF	S	No[27]	No upregulation[27]	MAIT: weak activation[27]	NT
	2-hydroxy-1-naphthaldehyde	2-OH-1-NA	S	Yes[27]	Moderate upregulation[27]	MAIT: inhibition[27]	NT
	3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl]formamido)propanoic acid	DB28	S	No[72]	Downregulation[72]	MAIT: inhibition[72]	NT
Synthetic 5-OP-RU/5-A-RU analogues	JYM72	-	S	Yes[26,47]	Potent upregulation[47]	MAIT: moderate activation	Yes[47]
	MR1 antigen analogue-tetramethylrhodamine	MAgA-TAMRA	S	Yes[75]	Upregulation[75]	MAIT: inhibition[75]	NT
	Ribityl-less analogue	-	S	Yes[26]	Potent upregulation[26]	MAIT: weak activation[26]	NT
	5-A-RU prodrug compound 10	5-A-RU prodrug	S	Yes[64]	NT	MAIT: activation[64]	NT
Uncharacterized ligands	Mammalian tumor-derived	-	N	Some no[41,42], some yes[40]	NT	MAIT: no activation[40,41] Atypical MR1T: activation (some)[40,41]	NT
	Microbial-derived (*Streptococcus pyogenes*)	-	N	NT	NT	MAIT: weak activation[60] Non-canonical MAIT: activation (some)[60]	NT

^MAIT refers to TRAV1-2^{+} typical MAIT cell population (*). NT: Not tested. #Comparing the potency of ligands is made difficult due to differences in assays across studies; however, where direct comparisons exist their relative potencies have been described.

989 **Table 2: Protein regulators of the human MR1 presentation pathway.** Proteins are divided into
990 MR1-binding or indirect regulators where binding has not been shown, not detected (ND) or not tested
991 (NT). The function of each protein on MR1 antigen (Ag) presentation is described from studies where
992 each was knocked out (KO) or knocked down (KD).

Protein/complex	Gene (human)	Primary subcellular location	Function	MR1 binding	Role in MR1 antigen presentation
MR1 binding accessories					
β-2-microglobulin	*B2M*	ER-Golgi, plasma membrane	Essential light chain of MHC-I and MHC-I-like molecules including MR1[77]	Yes[75]	KO prevents MR1 antigen presentation[16,75]
Tapasin	*TAPBP*	ER[128]	MHC-I chaperone and peptide editor[129]	Yes[75]	KO reduces MR1 protein stability, abundance, and antigen presentation[75]
TAPBPR	*TAPBPR*	ER-Golgi[88]	MHC-I chaperone and peptide editor[88]	Yes[75]	KO (along with Tapasin) reduces MR1 protein stability, abundance, and antigen presentation[75] Widens the antigen binding cleft[94]
AP2	*AP2A1, AP2M1, AP2S1, AP2B1*	Plasma membrane[130,131]	Recruits plasma membrane proteins for clathrin-mediated endocytosis[131,132]	Likely[67]	KO of *AP2A1* decreases MR1 endocytosis and recycling, increases half-life and antigen presentation[67]
Indirect regulators – MR1 binding not detected					
ATP13A1	*ATP13A1*	ER[84]	Translocase to remove mitochondrial proteins[84]	No[75,83]	KO reduces MR1 protein stability, abundance, and antigen presentation[83]
Ras-related protein Rab-6a	*RAB6A*	Golgi[133,134]	Intra-Golgi transport[133,134] Golgi-ER retrograde transport[135] Required for Golgi structure[135] Required for endosome to Golgi retrograde transport[136,137]	ND[75]	KD impairs presentation of endosomal Ag[69] and shows altered MR1 cellular distribution[119]
Syntaxin-4	*STX4*	Plasma membrane[138]	Endosome/granule to plasma membrane transport[138,139]	ND[75]	KD impairs presentation of extracellular Ag[116]
Syntaxin-16	*STX16*	Trans Golgi network[137]	Endosome to Golgi retrograde transport[137,140]	ND[75]	KD impairs presentation of extra- and endosomal Ag[116]
Syntaxin-18	*STX18*	ER[141,142]	Golgi-ER retrograde transport[141,142]	ND[75]	KD impairs presentation of extra- and endosomal Ag[116]
Vesicle associated membrane protein 2	*VAMP2*	Endosomes[143,144]	Endosome to plasma membrane transport[143]	ND[75]	KD impairs presentation of extra- and endosomal Ag[116]
Vesicle associated membrane protein 4	*VAMP4*	Trans Golgi network[145]	Required for Golgi structure[145] Endosome to Golgi retrograde transport[137]	ND[75]	KD impairs presentation of endosomal Ag[69]
Viral immunoevasins that affect MR1					

US3	*Us3*	-	Herpes simplex virus-1 (HSV-1) kinase that modulates several host cell processes, downregulates MHC class I and CD1d surface expression[146,147].	NT	Expression in cells reduces MR1 surface expression[102]. Deletion in HSV-1 reduced the impact on surface MR1[102].
US9	*Us9*	-	Human cytomegalovirus (HCMV) factor shown to target another MHC class I-related protein[148].	Yes[103]	Expression in cells reduces total cellular levels of MR1[103]. Deletion in HCMV did not prevent surface MR1 downregulation[103].
ORF66	*Orf66*	-	Varicella zoster virus (VSV) kinase, downregulates MHC class I[149].	NT	Expression in cells reduces MR1 surface expression[101]. Deletion in VSV did not prevent surface MR1 downregulation[101].

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Figure Legends

Figure 1: Proposed immune outcomes for MR1 presentation of metabolite antigens *in vivo*. A. Vitamin B-related antigen (VitBAg) is produced by yeast and most bacteria. It can reach the thymus from microbes on peripheral tissues and is presented by MR1 on double-positive thymocytes for the positive selection and development of MAIT cells. **B.** VitBAg released by commensal microbes at barrier tissues such as the skin is presented by MR1 and may recruit MAIT cells to this location and promote MAIT cell wound healing phenotype, although questions remain how important MR1 is in this process. **C.** During infection, VitBAg from extracellular or intracellular pathogens is presented by MR1To induce cytolytic killing of infected cells and the release of inflammatory mediators. **D.** Tumors can present different antigen on MR1 which induces their killing and release of cytokines by MR1-restricted T (MR1T) cells.

Figure 2: Major classes of ligands presented by MR1. A. MR1 antigens are derived from the riboflavin biosynthesis pathway that occurs within microbes (blue). The intermediate metabolite 5-A-RU can spontaneously react with small metabolites such as methyl glyoxal or glyoxal and give rise to the potent pyrimidine antigens 5-OP-RU or 5-OE-RU. These unstable molecules can condense to ribityl lumazines including RL-7-Me or RL. **B.** Additional ribityl lumazine antigens differ from side groups on the bicyclic lumazine ring. **C.** The folate-related MR1 ligands are the formyl pterins. **D.** A range of novel MR1 ligands include drugs and synthetic molecules with diverse structures. Side groups that form the Schiff base with MR1 are shown within white boxes.

Figure 3: The MR1 trafficking pathway and associated cellular machinery. A) In the steady state, where antigen is absent, MR1 resides in the ER-Golgi compartment stabilized by Tapasin or TAPBPR (1). Tapasin binds to either free MR1 heavy chains (HC) or MR1-β_2m dimers and may recruit MR1-β_2m to the peptide loading complex (PLC). The translocase ATP13A1 is located here in the ER and required for the cell to maintain a stable pool of MR1. Genetic screens have also identified STX18, VAMP4 and RAB6, which maintain the ER-Golgi compartment as and are important for the maintenance of the MR1 pool and its trafficking to the plasma membrane (2). **B**) In the presence of VitBAg, for example at barrier tissues or during infection with microbes, VitBAg is taken up by cells by unknown mechanismns; this may be directly from outside the cell or from phagosomes (3). This accesses the ER by an unknown

mechanism (4) and loads onto MR1, which may be facilitated by (5). VitBAgs such as 5-OP-RU form a covalent bond to the K43 residue in the antigen binding cleft of MR1. MR1-VitBAg complexes then traffic through the secretory pathway (6) to the plasma membrane for display to MAIT cells (7). After several hours, MR1 is recognized by the AP2 complex and internalized into early endosomes, where a small portion can recycle back to the cell surface (8). MR1 can exchange its cargo for an alternate ligand at the surface (9) or within endosomes (10). The majority of internalized MR1 molecules are subsequently degraded within lysosomes. **C**; structure of MR1 cleft (from PDB ID: 4nqc).

Figure 4: An intracellular pool of ligand-receptive MR1 molecules enables a strong antigen presentation signal. (**A**) Cells with high expression of MR1 have an abundant pool of ligand-receptive MR1 ready to capture VitBAg in the ER. **B**) Cells with low expression of MR1 or that lack Tapasin and TAPBPR, or ATP13A1, or have a dysregulated ER-Golgi compartment, have a depleted pool of ER-resident MR1. Upon exposure to VitBAg, MR1-high cells can display more MR1-VitBAg complexes at the cell surface than MR1-low cells, leading to an enhanced antigen presentation capacity.

Box 1: Nomenclature and functional diversity of MR1-restricted T cells

MAIT cells were discovered in the 1990s as a population of "preset" T cells with distinct features including the expression of a highly conserved TCRα chain, which contains TRAV1-2 gene segments joined to a limited number of TRAJ segments (TRAJ33/12/20)[31,32]. MAIT cells also undergo a unique developmental pathway in the thymus that is characterized by expression of the transcription factor PLZF[16,18,22]. In 2003 it was found that MR1 is their restricting MHC (like) molecule[17] and in 2013, that they recognize VitBAg ligands[13]. Since then, we have come to appreciate that there are T cells that recognize MR1 but do not fit with the canonical definition of 'MAIT cells'. These are much less abundant, express a different TCR, do not always follow the same developmental pathway, and, crucially, recognize other ligands. They may also be functionally distinct. For these reasons, the new term *MR1-restricted T (MR1T)* has been proposed to encompass MAIT and other MR1-restricted T cells. Three major classes of MR1T cells have been defined[20,21], although it is likely more subtypes will be described as new discoveries reveal further heterogeneity within the MR1T cell family:

- *MAIT* cells have the features described above, can be labelled with MR1+VitBAg tetramers and represent 1-10% of T cells in human blood[24,31].
- *Non-canonical MAIT* cells have some but not all the definitory properties of MAIT cells. They express a TRAV1-2$^-$ TCR but express PLZF and express similar phenotypic markers (such as CD161, CD44 and IL-18R) to MAIT cells. They recognize 5-OP-RU but also other ligands, some still undefined[11,60], and are rare (0.001-0.01% of blood T cells[21]).
- *Atypical MR1T* cells are the least abundant type of MR1T cells (up to 0.04% of blood T cells[41]). They express diverse TCRs, recognize non-VitBAg ligands including yet-undefined tumor antigens[40-42], and lack PLZF expression indicating absence of the innate-like developmental program followed by MAIT cells[21]. They may be conventional MHC class I-restricted CD8$^+$ T lymphocytes that cross-react with MR1-antigen complexes.

Box 2: MR1 evolution and conservation

MR1 is the most conserved antigen-presenting molecule, with 90% gene similarity in the α1-2 domains between humans and mice[150-152]. It is monogenic and is often described as monomorphic. This contrasts with classical MHC-I and -II, which are polygenic and among the most polymorphic of all human genes[153]. The allelic variants of MHC-I- and -II bind different peptidomes[154] but can all be considered "equally functional" because all variants contribute to selection of a fully functional T cell repertoire that protects against most challenges. Recent reports have described genetic variations in human MR1[155],[156]. Does this challenge its consideration as monomorphic? The question is important because if MR1 is conserved in the population, MR1T cell therapies may be applicable to any patient, unlike "classical" T cell-based approaches that require tailoring to the patient's MHC haplotype[154].

An analysis of a small cohort (56 donors) found that the prototypical *MR1*01* sequence is very common (75% frequency)[155]. Six human MR1 variants were found with at most two amino acid differences, caused by 1-3 single nucleotide polymorphisms (SNPs)[155]. In contrast, MHC-I alleles exhibit ~20 nucleotide differences in just the antigen-binding domains[157]. Only two MR1 variants have been shown to vary functionally from MR1*01. Firstly, a SNP that confers increased susceptibility to tuberculosis[156], but this is in an intron and predicted to influence MR1 transcription[156]. Secondly, a SNP that results in the Arg residue at position 9 to be mutated to histidine (R9H)[52,155] prevents the mutant MR1-R9H molecule from presenting the microbial ligand, 5-OP-RU. A patient homozygous for R9H lacked detectable MAIT cells[52]. This indicates that the mutation may be deleterious and therefore subject to negative selection pressure.

The few studies on MR1 genetic diversity among the human population are limited and deeper investigation may reveal greater variation. However, as it stands currently, MR1 appears to be remarkably conserved – even between species – and can be considered monomorphic, features that provide important clues to its function. Evolution has maintained the amino acid sequence of MR1 and its resulting function[158] and has evolved more slowly than MHC-I and other MHC-like genes[159]. Equally striking, MR1 has co-evolved with the MAIT cell TCRα gene, *TRAV1*; in species where *TRAV1* was lost, *MR1* was also lost or underwent significant mutations[159]. What is the driving force of this conservation? The polymorphism of classical MHC-I is an example of host-pathogen coevolution, as both adapt to present, or avoid presentation, of a

changing pathogen antigen landscape. The inverse argument applies for MR1 and its recognition by the MAIT TCR; the conservation of this system implies that it is adapted to detecting a limited number of ligands that are essential for the life of microbes and hence cannot vary[22,159]. The VitBAg 5-OP-RU is an example of such fundamental 'building block' of microorganisms.