Recognition of Vitamin B precursors and byproducts by Mucosal Associated Invariant T cells

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Abstract

Vitamin B2 (riboflavin) is essential for metabolic functions and is synthesized by many bacteria, yeast and plants, but not by mammals and other animals, which must acquire it from the diet. In mammals, modified pyrimidine intermediates from the microbial biosynthesis of riboflavin are recognized as signature biomarkers of microbial infection. This recognition occurs by specialized lymphocytes known as Mucosal Associated Invariant T (MAIT) cells. The Major Histocompatibility class I-like antigen presenting molecule, MR1, captures these pyrimidine intermediates, but only after their condensation with small molecules derived from glycolysis and other metabolic pathways to form short-lived antigens. The resulting MR1-Ag complexes are recognized by MAIT cell antigen receptors (αβ TCRs) and the subsequent MAIT cell immune responses are thought to protect the host from pathogens at mucosal surfaces. Here we review our understanding of how these novel antigens are generated and discuss their interactions with MR1 and MAIT TCRs.

MAIT cells

T cells are lymphocytes that mediate a range of immune functions such as killing infected host cells and producing cytokines and other factors that regulate immunity and inflammation. T cells generally recognize peptide antigens (Ags) or lipid-based Ags complexed to specialized Ag presenting molecules, MHC and CD1, respectively, that interact with an Ag-specific αβ T cell receptor (TCR) (1,2). An expansive repertoire of TCRs is generated by the random rearrangement of V, D and J gene segments and by pairing of TCR α- and β-chains, allowing specific recognition of a diverse range of Ags. Mucosal Associated Invariant T (MAIT) cells are a specialized subset of αβ T cells originally identified in CD4 CD8 (double negative, DN) human blood lymphocytes expressing a dominant invariant TCR α-chain gene rearrangement, TRAV1-2-TRAJ33 (based on IMGT, or Va7.2-Ja33 based on Arden nomenclature (3)) with less frequent usage of TRAJ12 and TRAJ20 (4-7). The MAIT TCR α-chain contains a complementarity-determining region (CDR) 3 loop of constant length with two variable amino acids in the V-J junction (8) located at the base of this loop (9,10). MAIT cells are evolutionarily conserved in mammals with the canonical TCR α-chain comprised of homologous TRAV and TRAJ
elements also found in mice, cattle (Vα19Ja33) (11) and sheep (12). TRAV1-2 is not exclusive to MAIT cells, with some MHC-I restricted T cells (13,14) and CD1b-restricted germline-encoded, mycolyl lipid–reactive (GEM) T cells also using this TRAV gene segment (15). The β-chain usage of MAIT cells has no apparent restrictions in Jβ usage, but there is a bias towards use of human TRBV20 and TRBV6 segments (or Vβ2 and Vβ13 based on Arden nomenclature) and, similarly in mice, to TRBV19 and TRBV13 (or Vβ6 and Vβ8 based on Arden nomenclature), both being the murine orthologous segments of human TRBV6 (7,8,11). Furthermore, recent TCR sequencing revealed a marked oligoclonality of MAIT TCR CDR3β regions and a bias in the length of mostly between 11 and 14 amino acids (7).

**MR1 presentation of antigens derived from vitamin B2 or B9 synthesis**

MAIT cell development and Ag-specific activation depends on expression of the Ag-presenting molecule MR1 [MHC related protein-1 (MR1)] (16), a major histocompatibility complex (MHC) class I–like molecule (17) that assembles with β2-microglobulin to form a heterodimer (18,19). MR1 is monomorphic (20) and is the most highly conserved MHC-like gene across diverse mammalian species with 90% sequence identity between mouse and humans (21,22) allowing for considerable species cross-reactivity of MAIT cells (23).

Until recently, the exact nature of the Ag presented by MR1 to T cells was unknown. It was initially postulated that the monomorphic nature of MR1 and the constrained MAIT TCR usage reflected a limited diversity in MR1 ligands recognised by MAIT cells in a pattern-recognition like manner (24). Notably, although human MAIT cells respond to a wide variety of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *S. epidermidis*, *Salmonella typhimurium*, *Mycobacterium smegmatis*, *M. tuberculosis*, *M. abscessus*, *Candida albicans*, *C. glabrata* and *Saccharomyces cerevisiae*, they are not activated by viruses and certain strains of *Listeria*, *Enterobacteria* and *Streptococci* (4,25-27). The canonical pathway of riboflavin biosynthesis involves a number of enzymes essential for ligand production (Figure 1A) and those bacteria that do not activate MAIT cells have defects in the riboflavin synthesis pathway (26), consistent with derivation of MR1 ligands from riboflavin synthesis. In some bacteria the riboflavin synthesis pathway is regulated by an operon (Rib) such that the presence of flavin mononucleotides, or riboflavin itself, inhibits riboflavin synthesis and impairs generation of ligands that activate MAIT cells (28,29). Moreover, in two bacterial strains, *Salmonella enterica* serovar Typhimurium and *Lactococcus lactis*, MAIT cell activation was dependent on the enzymatic production of 5-amino-6-D-ribitylaminouracil (5-A-RU), an intermediate in riboflavin biosynthesis that is present in diverse bacteria and yeast as well as plants (29). Mutations in enzymes essential to the production of 5-A-RU, but not enzymes required later in riboflavin synthesis (Figure 1A), prevented the production of activating MAIT cell ligands by these bacteria (29). This finding was recently verified in mutants of *E. coli* (30). While 5-A-RU plays an important role in MAIT cell activation, MR1 could not be refolded efficiently with 5-A-RU alone (29,31). Despite the link between MAIT cell activation and the biosynthesis of vitamin B2, the first MR1-binding ligand to be identified was 6-formylpterin (6-FP), a photodegradation product of vitamin B9 (folic acid) (26). It was identified by liquid chromatography-mass spectrometric (LC-MS) analysis of the eluted material from recombinant MR1 molecules assembled in folic acid-containing media (26). However, MR1-6-FP complexes did not activate MAIT cells, leading us to a wider search for the natural activating ligand (26). When recombinant MR1 was loaded with filtered bacterial cultures of *S. Typhimurium* grown in folate-deficient media, LC-MS indicated that the main chemical component from the eluted material had a molecular formula of C13H18N4O7 (26). A search for compounds structurally related to riboflavin biosynthetic intermediates matching this formula included (i) a dihydrogen-reduced product of 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-7-HO, C10H14N4O2), a metabolite of the key intermediate 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-DiMe, C13H18N4O7, Figure 1A and B) and (ii) the dihydrogen-reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-OH, C13H18N4O7), the latter of which had no known link to riboflavin
biosynthetic intermediates apart from structural similarity. Indeed, all three compounds (RL-6, 7-diMe, RL-6-Me-7-OH and rRL-6-CH$_2$OH) activated MAIT cells in an MR1-dependent manner in cellular assays, albeit with varied levels of potency (26).

MR1 assembled with RL-6-Me-7-OH sufficiently well in vitro for the MAIT TCR-MR1-RL-6-Me-7-OH structure to be solved by X-ray crystallography (9), although the recovered complexes were very unstable and made tetramer production challenging (6). At this point, rRL-6-CH$_2$OH seemed to be the most potent activator of MAIT cells in vitro and ex vivo (26). However, wild type MR1 could not be refolded with rRL-6-CH$_2$OH so that a crystal structure could not be obtained for MR1 complexed with rRL-6-CH$_2$OH. A low-resolution (3.3Å) crystal structure of a human MAIT TCR and humanized bovine MR1 complexed with a heterogeneous extract from *E. coli* was later reported but the nature of the bound Ag(s) remains unclear (32). Therefore we took a genetic and biochemical approach to identifying the nature of the ligands captured by MR1 from the riboflavin synthesis pathway in bacteria.

**Formation of MAIT cell ligands through the interaction of different metabolic pathways**

A key step in riboflavin biosynthesis is the condensation of 5-A-RU with 3,4-dihydroxy-2-butane-4-phosphate (3,4-DH-2-B-4-P) to generate RL-6, 7-DiMe, (Figure 1A). This reaction proceeds via a putative intermediate, 5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU), which spontaneously undergoes ring closure via dehydration to form RL-6, 7-DiMe (33,34) (Figure 1B). This reaction can be catalyzed by lumazine synthase (RibH), but RL-6, 7-DiMe can also be generated in the absence of an enzyme (33,34). Since 5-MOP-RU and the related compound 5-(2-oxopropylideneamino) 6-D-ribitylaminouracil (5-OP-RU), which spontaneously undergoes ring closure via dehydration to form RL-6, 7-DiMe (33,34) (Figure 1B). This reaction can be catalyzed by lumazine synthase (RibH), but RL-6, 7-DiMe can also be generated in the absence of an enzyme (33,34). Since 5-MOP-RU and the related compound 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) differ only by a single methyl group, we conceived that 5-OP-RU might be formed as a short-lived Ag via the analogous condensation of 5-A-RU with methylglyoxal, en route to cyclization to the corresponding lumazine 7-methyl-8-D-ribityllumazine (RL-7-Me) (Figure 1B). In attempts to generate potential MAIT cell activators, we therefore chemically combined the key riboflavin biosynthetic intermediate 5-A-RU with glyoxal and methylglyoxal, small molecules abundantly formed in a number of metabolic pathways including glycolysis (35). When these reaction mixtures were used to refold MR1, the ligands identified in the cleft were surprisingly the chemically unstable pyrimidine intermediates 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-OP-RU, instead of the corresponding and relatively more stable ribityl lumazines, 8-D-ribityllumazine (RL) and RL-7-Me, respectively. These Ags, 5-OP-RU and 5-OP-RU, are normally unstable in water, but were encapsulated and stabilized by MR1 through Schiff base formation (see below) and an extensive hydrogen bonding network as evidenced in high resolution (2.1-2.6 Å) crystal structures (29). Both Ags activated MAIT cells and formed stable MR1 tetramers that stained MAIT cells with great specificity in the case of both human (29) and mouse MAIT cells (36). Furthermore, they were identical to the ligands recovered from recombinant MR1 assembled with supernatant from *S. Typhimurium, L. lactis*, and *E. coli*, as determined by high resolution LC-MS (29). Other researchers have since also confirmed the identity of these MAIT cell activating ligands, 5-OP-RU and 5-OP-RU (30). The structural features of their binding revealed the basis for the capture of these intermediates by MR1 as discussed below (37).

By contrast, 5-A-RU combined with 2,3-butanedione did not activate MAIT cells or refold with MR1 though we are uncertain whether the creation of 5-MOP-RU en route to RL-6, 7-DiMe occurs efficiently. This inability of MR1 to bind to an essential intermediate of riboflavin biosynthesis may be critical in directing MAIT cell activation.

**Structural basis of MR1-Antigen binding and MAIT cell TCR recognition**

Consistent with its sequence similarity to an MHC-I molecule, MR1 has a structure that overall is analogous to MHC-I, but where the Ag is contained within an aromatic cradle formed between the α1- and α2-helices sitting atop an antiparallel β-sheet (Figure 2A) (26). The cradle is lined with both charged and hydrophobic residues, of which a large number are aromatic, thus providing an ideal environment for the capture of small molecules (26). A number of high-resolution MAIT TCR-MR1 ternary structures determined by X-ray crystallography has allowed the
examination of the intermolecular interactions involved in capture and presentation of this novel class of Ags by MR1 and revealed a number of interesting features (2,7,24,28,30).

The most unusual feature of MR1 was identified from the initial MR1-6-FP structure and subsequent analysis, where the Ag was covalently bound to MR1 through a Schiff base formed between the formyl group of 6-FP and Lys43 of MR1 (Figure 2B) (26). This covalent bond demonstrates a strong association between MR1 and the Ag, despite its inability to activate MAIT cells. The Lys43 residue is located at the base of the cleft and thus the small 6-FP molecule is also buried deep within the comparatively large solvent accessible MR1 cavity (Figure 2B) (26). Consequently, the crystal structure of MR1-6-FP bound to MAIT TCR, demonstrated the relative inaccessibility of the compound in this location for TCR recognition (9). The TCR bound orthogonally to the main axis of the Ag-binding cleft and centrally over the cradle placing the variable regions of the TCR on the surface of MR1 immediately above the compound (Figure 2A) (9).

The Tyr95 residue from the CDR3α loop extended down into the cleft but only formed a single indirect link between 6-FP and the TCR, through a water-mediated hydrogen bond (Figure 2B) (9). The crystal structure of a MAIT TCR bound to complexes of MR1-RL-6-Me-7-OH provided initial insight into the mechanism of MAIT cell activation, noting that RL-6-Me-7-OH only weakly activated MAIT cells (9). In this structure, the TCR was bound to MR1 in an identical fashion to the non-activating MR1-6-FP complexes, but made a direct hydrogen bond with the ribityl moiety of the Ag through the highly conserved Tyr95 (Figure 2B), explaining the differential MAIT cell responses. Notably, RL-6-Me-7-OH cannot form a Schiff base with MR1, as it does not have a carbonyl group. The larger RL-6-Me-7-OH Ag also occupied a more expansive region of the cavity making numerous contacts within the cleft in order to correctly orient the compound for T cell recognition in the absence of Schiff base bond formation (Figure 2C). The structures of MAIT TCRs complexed with MR1-5-OE-RU and MR1-5-OP-RU showed the same orientation of the ribityl moiety so that the Ag could also form a direct contact with Tyr95 of the MAIT TCR (Figure 2B) (9). However, in contrast to RL-6-Me-7-OH binding to MR1, both 5-OE-RU and 5-OP-RU were able to form a Schiff base bond with Lys43 through a reactive carbonyl group (29). Furthermore, the CDR3β loop of the TCR β-chain now made an additional contact with both pyrimidine adducts through its own water-mediated hydrogen bond (Figure 2B) (29). Affinities between MR1-5-OP-RU or MR1-5-OE-RU complexes and MAIT TCRs measured by surface plasmon resonance were comparable to conventional TCRs recognizing pMHC complexes (Kd ~ 0.5-10 µM) (31).

Notably, although rRL-6-CH2OH could not be refolded with wild type MR1, it could be loaded into pre-folded MR1 molecules containing a Lys43Ala mutation that prohibits formation of a Schiff base with Ag. This crystal structure also revealed that the trapped ligand was the monocyclic 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (Figure 2B) (29). This bound ligand had an LC-MS/MS fragmentation pattern that was indistinguishable from rRL-6-CH2OH (28,29) and is primed for cyclisation via intramolecular condensation of the free amine with the ketone. Consistent with these findings, a trace amount of 5-OP-RU was often present in synthetic samples of rRL-6-CH2OH (Figure 3 and unpublished). The structure of a MAIT TCR-MR1(Lys43Ala)-5-OP-RU complex clearly demonstrated that the interactions within the MR1 cleft were sufficient to stabilize the 5-OP-RU pyrimidine adduct and prevent ligand cyclization without the contribution of Lys43 to capture the reactive carbonyl group via Schiff base formation (Figure 2C) (29). Although the structure confirmed that the covalent Schiff base interaction is not required by MR1 for Ag capture or activation of MAIT cells, MR1-Ag stability was reduced markedly in the absence of the Schiff base (17ºC lower half maximum melting temperature by thermostability) (31).

**MAIT cell antigen diversity**

Whilst 5-OP-RU and 5-OE-RU are potent MAIT cell Ags, the full range of naturally produced 5-A-RU adducts has not yet been defined. It is possible that other small molecules in addition to glyoxal and methylglyoxal may also arise as physiological byproducts potentially generating distinct variation in ligands in different microbes, or metabolic stages of the microbe and host. For example,
whereas 5-OE-RU was the dominant species in the supernatant of E. coli, this was a subdominant species in supernatant of S. Typhimurium, indicating that the bacterial source can impact on the relative abundance of the two distinct ligands (29).

The variability of MAIT TCR β-chain usage, and the original discovery of several MAIT cell ligands recognized by MAIT cells, suggest there may be additional natural Ags that stimulate MAIT cells (26). As discussed earlier, in the dominant MAIT TCRα, residue Tyr95 forms a hydrogen bond with the ribityl moiety of activating ligands that is pivotal in MAIT cell activation (6,9,10,31,38). However, other less frequent TRAJ regions have been sequenced and tentatively assigned to MAIT cells, two of which do not encode Tyr95 (TRAJ9, TRAJ39) (25). They may not represent functional MAIT TCRs, perhaps reflecting incomplete allelic exclusion at the TRA locus, but alternatively, it is possible that they are either reactive with known activating Ags in a manner distinct to MAIT TCRs encoding TRAJ Tyr95, or that they recognise novel MAIT cell Ags. Structural studies have shown that MAIT TCR heterogeneity, especially in the CDR3β loop can fine-tune MR1 recognition in an Ag-dependent manner (31), highlighting the possibility that MAIT cells might have the capacity to discriminate between diverse ligands in a MAIT cell subset specific manner. Gold et al. have recently observed distinct MAIT TCR repertoire mobilization in response to diverse pathogens that could reflect the existence of discrete pathogen associated Ags presented by MR1 (25). This possibility is yet to be confirmed with biochemical evidence. On the other hand, the limited variation in the MAIT TCR α-chain and oligoclonality in the CDR3β loop partly reflect structural requirements for MR1 recognition since both regions are critical as illuminated in crystal structures and mutagenesis analysis (9,10,38). Another contributing factor to the limited MAIT TCR repertoire might be preferential TCR rearrangements and α- and β-chain pairing. To this end it has been confirmed that the canonical TRAV1-2-TRAJ33 amino acid sequence is produced efficiently from multiple TCR nucleotide sequences that can arise from multiple recombination events, a process referred to as convergent recombination (39). Interestingly, in Vα19i Cα′MR1+ mice a significant population of ‘MAIT-like’ T cells develop, apparently selected by classical MHC-I molecules but reactive to MAIT Ags presented by MR1+ Ag presenting cells (40). This raises the possibility that the variability of MAIT TCR β-chain and Jα usage might also be driven by avoidance of self-reactivity to polymorphic MHC-I and MHC-II molecules by the relatively fixed MAIT TCR.

The X-ray crystal structures of MR1-Ag complexes also raise the possibility of additional, novel MR1 Ags. Thus, all structures to date have shown the Ag to be bound within the MR1 A’-pocket (Figure 2D, equivalent to the MHC-I pocket that binds the N-terminal peptide residue), which contains both Lys43 for Schiff base formation and the residues that interact directly with the ribityl moiety. However, the F’-pocket (equivalent to the MHC-I pocket that binds the C-terminal residue of peptides), which is shallower than the A’ pocket, has already shown a predisposition to presenting small molecules (Figure 2D). A number of the published MAIT TCR-MR1 structures deposited to the PDB contain the buffer compounds, 1,3-bis(tris(hydroxymethyl)methylamino)propane or glycerol (eg. 4PJE and 4PJE), used in the crystallisation conditions, located within this cleft (31). This molecule makes a number of hydrogen bonds with charged residues in the F’ pocket (Figure 2E) and, while they are unlikely to be MAIT cell Ags themselves and are relatively distant from the canonical MAIT TCR docking region, it does provide a tantalizing glimpse of what naturally occurring MR1 ligands within this F’-pocket may resemble.

It is possible, but not obvious, that distinct MR1 ligands might be derived from additional microbial pathways other than riboflavin biosynthesis. However, the genetic correlation between the riboflavin biosynthetic pathway, ligand production and MAIT cell activation suggests this is a dominant source of Ag. This in turn, implies that the basic scaffold of any additional microbial Ags will derive from 5-A- RU. Indeed, tetramers of MR1-5-OP-RU or MR1-5-OE-RU bind to most if not all MAIT cells (6), suggesting that 5-OP-RU and 5-OE-RU represent universal and highly potent MAIT cell Ags.

**MR1 expression and ligand capture**
MR1 transcripts and intracellular protein are ubiquitously expressed (17,21), whilst MR1 is expressed at low levels at the cell surface and is more readily detectable in the presence of ligand (26,31,41). Studies prior to the discovery of MR1 ligands showed that intracellularly MR1 is primarily found in the endoplasmic reticulum where it associates with all known members of the peptide loading complex including calnexin, calreticulin, ERp57, TAP, and tapasin even though not all residues known to interact with the loading complex are present in MR1, thus pointing towards a novel manner of loading complex interaction (19). However, MAIT cell activation by endogenous ligand was shown to be independent of chaperoning by the MHC loading complex (16,42). Instead the endocytic compartment including MHC class II chaperones Ii and DM, which promote endosomal trafficking, were shown to be important for MAIT cell activation in this system (42). Notably MR1 can present both, extracellular (supernatant) and infection-related Ag. The precise mechanisms by which bacterial Ags are loaded onto MR1 and the location of this event in each case remain to be understood. It is also still unclear if MR1 Ag presentation is mediated by a specialised Ag presenting cell in the periphery, as both myeloid cells as well as non-myeloid cells, such as epithelial cell lines including A549(4), BEAS-2B (43) and HeLa (44), can act as antigen presenting cells in vitro. Both 6-FP (26), and its synthetic analogue acetyl-6-formylpterin (Ac-6-FP) (Figure 4) (31) potently upregulate cell surface expression of MR1 and act as competitive inhibitors of MAIT cell activation by 5-OP-RU, the latter being the superior competitive inhibitor. When comparing the kinetics of MR1 upregulation, Ac-6-FP induced a more rapid and more prolonged increase in MR1 levels at the cell surface and higher maximum surface expression levels of MR1 were reached (31). The difference could reflect the lower stability of MR1-6-FP as compared to MR1-Ac-6-FP (8°C lower half maximum melting temperature by thermostability) (31). This difference in stability was associated with additional hydrogen bonding between the acetyl group of Ac-6-FP and Arg94 of MR1, although we cannot rule out effects related to the intrinsic stability of the ligands themselves or their relative cellular uptake. Additional analogues of 6-FP that upregulate MR1 and competitively inhibit MAIT cell activation have recently been reported, namely the dimethyl acetals of 6-FP and Ac-6-FP (30), (Figure 4). Both possess these activities despite being unable to form a Schiff base with MR1, although partial hydrolysis of the acetal could generate an aldehyde capable of reacting with Lys43 (30). MAIT cells in human PBMCs and MAIT cell reporter cell lines are activated at nM concentrations of Ag ((31) and unpublished), suggesting that a small number of cell surface MR1-5-OP-RU complexes might be required for MAIT cell activation. Consistent with this notion, competitive inhibition of 5-OP-RU by the most potent inhibitor, Ac-6-FP, requires formation of stable MR1-antagonist complexes and 10^6-fold molar inhibitor excess for complete competitive inhibition of activation by 5-OP-RU (31).

In summary, MR1 has sufficient plasticity to accommodate a range of chemical entities, including those identified to date such as the bicyclic pterins (6-FP, Ac-6-FP), monocyclic pyrimidine derivatives (5-OP-RU, 5-OE-RU) and bicyclic lumazines (RL-6, 7-DiMe, RL-6-Me-7-OH) (9,26,29,31), all of which are derived from vitamin B2 (riboflavin) or B9 (folic acid) biosynthesis. It is not yet known whether distinct classes of ligands can be accommodated in the A’ versus the more solvent exposed F’ pockets of MR1. Further studies are in progress to map the full complement of potential MR1-binding ligands that can activate or inhibit MAIT cells, and to characterize the immunological and pharmacological consequences of MR1 recognition of small heterocyclic molecules and their subsequent presentation as Ags for MAIT cell activation. These ongoing studies promise to reveal new insights into the molecular basis of MAIT cell mediated immune responses in physiology and disease.
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References


was highly conserved in evolution. *Proceedings of the National Academy of Sciences of the United States of America* 106, 8290-8295


Figure legends

Figure 1. A) Schematic display of the Riboflavin biosynthesis pathway. ribH, lumazine synthase; X, hypothetical phosphatase. B) Chemical formation of pyrimidines and ribityllumazines from condensation of small metabolites with 5-A-RU. 5-A-RU: 5-amino-6-D-ribitylaminouracil, 3,4-DH-2-B-4-P: 3,4-dihydroxy-2-butanone-4-phosphate, 5-MOP-RU: 5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil, RL-6,7-diMe: 6,7-dimethyl-8-D-ribityllumazine, 5-OP-RU: 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil, RL-7-Me: 7-methyl-8-D-ribityllumazine, 5-OE-RU: 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil, RL: 8-D-ribityllumazine.
Figure 2. Structural characterization of MR1-binding ligands derived from vitamin B synthetic intermediates. A) Ternary structure of MR1 presenting 6-FP to MAIT TCR. Boxed area represents that shown in B. B) Contact between the MR1 bound antigens 6-FP, RL-6-Me-7-OH, 5-OP-RU or non-covalently bound 5-OP-RU, with the MAIT TCR. C) Contacts sequestering 6-FP, RL-6-Me-7-OH, 5-OP-RU or non-covalently bound 5-OP-RU within the MR1 binding cleft. D) Solvent accessible area within the MR1 binding cleft with the A’ and F’ pockets labeled. The CDR3α (yellow) and CDR3β (orange) of the MAIT TCR are shown. E) 1,3-bis(tris(hydroxymethyl)methylamino)propane bound within the F’ pocket of MR1 (from PDBID: 4PJX). All dashed black lines represent hydrogen bonds and red spheres represent water molecules.

Figure 3. Synthesis of rRL-6-CH$_2$OH and the possible formation of the byproduct 5-OP-RU.

Figure 4. Folic acid and its structurally related MR1 ligands.
Figure 1

**A**

- **Ribulose-5-phosphate**
- **3,4-Dihydroxy-2-butane-4-phosphate**
- **Riboflavin**
- **6,7-Dimethyl-8-D-ribityllumazine**
- **7-Hydroxy-6-methyl-8-D-ribityllumazine**

**B**

- **5-A-RU**
- **Pyrimidines**
- **Ribityllumazines**

**5-OE-RU**

**5-OP-RU**

**RL**

**RL-7-Me**
Figure 4

- Folic acid
- 6-formylpterin (6-FP)
- Acetyl 6-formylpterin (Ac 6-FP)
- 6-formylpterin dimethyl acetal
- Acetyl 6-formylpterin dimethyl acetal
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