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In Vitro and In Vivo Analysis of the Gram-Negative Bacteria–Derived Riboflavin Precursor Derivatives Activating Mouse MAIT Cells

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Mucosal-associated invariant T (MAIT) cells recognize microbial compounds presented by the MHC-related 1 (MR1) protein. Although riboflavin precursor derivatives from Gram-positive bacteria have been characterized, some level of ligand heterogeneity has been suggested through the analysis of the MAIT cell TCR repertoire in humans and differential reactivity of human MAIT cell clones according to the bacteria. In this study, using Gram-negative bacteria mutated for the riboflavin biosynthetic pathway, we show a strict correlation between the ability to synthesize the 5-amino-ribityl-uracil riboflavin precursor and to activate polyclonal and quasi-monoclonal mouse MAIT cells. To our knowledge, we show for the first time that the semipurified bacterial fraction and the synthetic ligand activate murine MAIT cells in vitro and in vivo. We describe new MR1 ligands that do not activate MAIT cells but compete with bacterial and synthetic compounds activating MAIT cells, providing the capacity to modulate MAIT cell activation. Through competition experiments, we show that the most active synthetic MAIT cell ligand displays the same functional avidity for MR1 as does the microbial compound. Altogether, these results show that most, if not all, MAIT cell ligands found in *Escherichia coli* are related to the riboflavin biosynthetic pathway and display very limited heterogeneity. *The Journal of Immunology*, 2015, 194: 4641–4649.

Mucosal-associated invariant T (MAIT) cells represent an abundant subset in human blood that recognizes microbial compounds presented by the nonclassical MHC-related 1 (MR1) molecule (1, 2). MR1 is highly conserved between species, indicating a strong evolutionary pressure and,

therefore, important function(s). MR1 is also nonpolymorphic, suggesting ligand(s) with limited diversity (2). The murine MAIT cell TCR is made up of the iVα19-Jα33 (iVα19) semi-invariant TCRα-chain associated with a limited set of Vβ segments (Vβ6 and Vβ8) (3). Because MAIT cells are extremely rare in conventional laboratory mouse strains, three groups generated iVα19 TCRα-transgenic (Tg) (single-Tg [STg]) mouse models (4–6). Although an MR1-dependent increased usage of Vβ6 and Vβ8 TCRβ segments is found in these iVα19-Tg mice, their repertoire remains polyclonal, even on a Cα$^{-/-}$ background, because endogenous TCRβ-chains are used (5, 6). We also generated iVα19Vβ6 Cα$^{-/-}$ double-Tg (DTg) mouse lines (6) that display a quasi-monoclonal repertoire.

MAIT cells are activated by coculture of epithelial cells overexpressing MR1 with a large variety of bacteria and yeasts (7, 8). The correlation between the presence of the riboflavin (vitamin B2) biosynthesis pathway in a given bacteria and their ability to activate MAIT cells suggested that the MAIT cell ligand(s) originated from this pathway (8, 9). Several compounds related to the riboflavin biosynthesis pathway were identified as putative MAIT cell ligands (9). Analysis of the MAIT cell–activating capacity of Gram-positive bacteria harboring inactivating mutations for the different enzymes of the riboflavin pathway identified 5-amino-ribityl-uracil (5-A-RU), an unstable precursor of riboflavin, as a key compound for the formation of MAIT cell ligand(s) (10). MR1-refolding experiments and structural analysis demonstrated the presence of unstable pyrimidine adducts that are bound and stabilized in the MR1 groove. They are generated by the nonenzymatic reaction of 5-A-RU with small metabolites (glyoxal and methyl-glyoxal) of the intermediary metabolisms (glycolysis in particular) (10). Although a *Salmonella*-derived MAIT cell ligand appears structurally similar to these adducts, it remains to be determined how the MAIT cell ligand(s) found in Gram-negative

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Abbreviations used in this article: 5-A-RU, 5-amino-ribityl-uracil; a.u., arbitrary unit; Cp-A, compound A; Cp-B, compound B; Cp-C, compound C; Cp-D, compound D; DHA, dihydroxyacetone; DN, double negative; DTg, double Tg; 6-FP, 6-formyl pterin; Glyx, glyoxal; iVα19, iVα19-Jα33; MAIT, mucosal-associated invariant T; MeG, methylglyoxal; MOI, multiplicity of infection; MR1, MHC-related 1; 5-N-RU, 5-nitroribitylaminouracil; rRL-6, reduced 6-(hydroxymethyl)-8-D-ribityllumazine; SPB, semipurified soluble bacteria; STg, single Tg; Tg, transgenic; WT3-m, WT3 cell stably overexpressing mouse MR1.

bacteria relates to the riboflavin biosynthesis pathway. Indeed, although the enzymes of the riboflavin synthesis pathway are grouped in a highly regulated operon in Gram-positive bacteria, these enzymes are dispersed in the genome of Gram-negative bacteria, and subtle differences in the nature or the abundance of the intermediary compounds that could be generated may occur.

It was suggested that human MAIT cells display different patterns of reactivity toward different microbes that all harbor a functional riboflavin biosynthesis pathway (*Mycobacterium smegmatis*, *Salmonella typhimurium*, and *Candida albicans*) (11). TCR repertoire analysis of polyclonal human Vα7.2$^+$ (TRAV1) CD8 T cells secreting TNF-α after stimulation by these microbes suggested a large diversity of TCRs because several Jαs and different TCRβ-chains were observed (11). In addition, some Vα7.2-expressing clones displayed a different pattern of reactivity toward the microbes mentioned above. However, in the absence of an anti–MR1–blocking experiment, it is not clear whether the reactivity of the clones was lymphokine mediated or classical MHC or MR1 restricted. These two sets of data (diversity of TCRβ repertoire of polyclonal TNF secreting Vα7.2$^+$ T cells and distinct patterns of bacterial reactivity by MAIT cell clones) led the investigators to suggest that a variety of microbial ligands would be recognized by distinct sets of MAIT cells expressing distinct TCRs (11). In contrast, mutagenesis experiments associated with structural studies identified crucial residues in the CDRs of the TCRα-chain that are required for Ag recognition by MAIT cells, regardless of Vβ–Jβ usage (12). These residues are conserved in mammals (13, 14). Although MAIT cell TCRs use preferential Vβ segments, mutagenesis of individual residues within the CDRβ regions indicates that the TCRβ-chain of the MAIT cell TCRs did not play an important role in Ag reactivity, consistent with the lack of TCRβ constraints (12, 15). Altogether, the structural data are consistent with a limited number of MAIT cell ligands that do not fill the MR1 groove as they leave empty the F′ pocket (16, 17). This opens the possibility of some level of complexity for MAIT cell ligands.

Notably, the structural data seen above were obtained through the exclusive use of human MAIT cell TCRs. Although murine MR1 tetramers loaded with riboflavin derivatives stain a large proportion of the T cells found in *i*Vα19-Tg mice, the possibility of additional ligands remains. Indeed, MAIT cells are protective in a variety of experimental infection models: MR1-deficient mice are more susceptible to *Mycobacterium abscessus* or *Escherichia coli* infections (8). MAIT cells contribute to the control of *Klebsiella pneumoniae* (18) and *Francisella tularensis* (19). Finally, no data are available with regard to the in vitro or in vivo activation of murine MAIT cells by bacterial or synthetic ligands.

In this study, we determined which steps of the riboflavin pathway in the Gram-negative bacteria *E. coli* are necessary for MAIT cell activation. We compared the activation of polyclonal or quasi-monoclonal murine MAIT cells using either synthetic MAIT cell ligand or semipurified bacterial fractions and found no evidence for ligand heterogeneity. For the first time, to our knowledge, we show direct in vivo activation of murine MAIT cells by natural bacterial ligand and synthetic compounds. Using compounds that bind to MR1, but do not activate MAIT cells, we show that the biologically active compound present in the Gram-negative bacterial fraction has the same avidity for MR1 as does the synthetic chemical compound. The compounds that do not activate MAIT cells but bind MR1 represent a new class of MAIT cell–activation inhibitors.

Materials and Methods

Strains, plasmids, and growth conditions

E. coli bacterial strains used in this study are available upon request. The strain DH5α (Bethesda Research Laboratories) was used as the host for propagation of plasmids. Riboflavin-requiring mutants were obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). The p*Trc*99A (20) and pUC4K (21) plasmid vectors were from Amersham Biosciences, and the p*Trc*His30 vector was described previously (22). The pKD3 and pKD46 plasmids used for gene-disruption experiments were kindly provided by B. Wanner (Department of Biological Sciences, Purdue University, West Lafayette, IN) (23). The different plasmids constructed in this work are listed in Supplemental Table IA, IB. Unless otherwise noted, cells were grown in 2YT medium (24) at 37°C. Riboflavin (20 μg/ml) was added when needed, and ampicillin, kanamycin, and chloramphenicol were used at 100, 50, and 25 μg/ml, respectively. Growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer.

Molecular biology techniques

PCR amplification of genes was performed in a Biomed Thermocycler 60 apparatus (B. Braun) using Expand High Fidelity polymerase (Roche). DNA fragments were purified using the Wizard PCR Preps DNA Purification System (Promega), and standard procedures for DNA digestion, ligation, and agarose gel electrophoresis were used (25). Plasmid purification kits were from Macherey-Nagel, and *E. coli* cells were transformed with plasmid DNA using the method of Dagert and Ehrlich (26) or by electroporation.

Construction of expression plasmids

The *ribA* gene was amplified from the DH5α chromosome using primers RibA1 and RibA2 (Supplemental Table IC), and the resulting material was treated with BamHI and HindIII and then ligated between the same sites of plasmid vector p*Trc*His30, generating plasmid pMLD410. The *ribB* gene was amplified using RibB1 and RibB2 primers, and the DNA fragment was cut by BspHI and HindIII and inserted between the compatible sites NcoI and HindIII of the p*Trc*99A vector, generating pMLD411. Similarly, the *ribC* gene was amplified using RibC1 and RibC2 primers, and the PCR product was cleaved with BspLU11I and HindIII and was ligated to the p*Trc*99A vector digested by NcoI and HindIII, generating the pMLD412 plasmid. In the case of *ribD*, the GTG initiation codon of the chromosomal gene sequence was replaced by an ATG in the expression vector: the gene was amplified using the RibD1 and RibD2 primers, and the PCR product was cut by BamHI and HindIII and inserted between the same sites of the p*Trc*His30 vector, yielding pMLD418. The *ribE* gene was amplified with RibE1 and RibE2 primers, and the resulting fragment was cleaved by BspHI and HindIII and inserted between the NcoI and HindIII sites of p*Trc*99A, generating pMLD415. Derivatives of these different constructs that expressed kanamycin resistance instead of ampicillin resistance were generated as follows: plasmids were linearized by ScaI, whose unique restriction site lies within the *amp*^{R} gene, and were ligated to a 1.3-kb HincII kanamycin resistance gene cassette originating from pUC4K. In all cases, the sequence of cloned inserts was controlled by DNA sequencing.

Inactivation of chromosomal ribABCDE genes

Genes involved in the riboflavin pathway were individually deleted and replaced by a chloramphenicol resistance cassette in the chromosome of strain BW25113. This was done using the classical procedure of Datsenko and Wanner (23) with pairs of oligonucleotides RibX-inact1 and RibX-inact2 (with X being A, B, C, D, or E; Supplemental Table IC). Because riboflavin is essential for growth and is normally not taken up by wild-type *E. coli* cells, the inactivation procedure was applied to cells carrying a wild-type copy of these genes *in trans* on an expression plasmid. Therefore, BW25113 (pKD46) cells also contained one of the plasmids pMLD419, pMLD420, pMLD421, pMLD424, or pMLD422 to allow inactivation of chromosomal genes *ribA*, *ribB*, *ribC*, *ribD*, or *ribE*, respectively. In all cases, chloramphenicol-resistant clones were isolated, and the deletion of the chromosomal *ribX* genes and their replacement by the Cm^R cassette was confirmed by PCR using appropriate oligonucleotides RibX-ext1 and RibX-ext2 (sequences are available upon request). These mutations were transduced into other genetic backgrounds of interest with phage P1 (24).

Mice

The mice used in this study were described previously (6). Briefly, *i*Vα19-Tg animals were on a Cα-deficient B6 background to avoid endogenous Vα expression. All Vβ6-Tg mice were generated on the C57BL/6 background, and *i*Vα19-Tg mice and MR1-deficient mice were backcrossed onto the C57BL/6 background for >10 generations. For in vivo injections, *i*Vα19-Tg mice were injected i.p. with 100 arbitrary units (a.u.) of the semipurified fractions (see below the definition of one unit) or with 100 nmol 5-A-RU plus MeG or PBS as a negative control. Eighteen hours

after injection, spleens were recovered, mechanically dissociated, and stained. All mice were housed in our accredited specific pathogen–free colony and genotyped by PCR or FACS staining, as appropriate. Live animal experiments were done in accordance with the guidelines of the French Veterinary Department and the ethical committee of the Curie Institute.

Cell preparation

Cell suspensions were prepared from spleen by mechanical disruption on cell strainers. Cells were cultured in DMEM + GlutaMAX supplemented with 10% FCS, penicillin and streptomycin, nonessential amino acids, HEPES, and sodium pyruvate (all from Life Technologies). For in vitro T cell activation, splenocytes were incubated with magnetic beads (anti-CD11c, anti-CD19, and anti-CD4) prior to magnetic separation using the MACS Pro System, according to the manufacturer's recommendations. This depletion allows for enrichment of MAIT cells. The mouse cell lines WT3 and WT3 stably overexpressing the mouse MR1 molecule (WT3-m) were described elsewhere (1).

Microbes, infection, and in vitro MAIT cell activation

Bacteria grown to stationary phase were washed and put in DMEM without supplement. Serial dilutions of bacteria were incubated with WT3 or WT3-m cells (1×10^5/well) in DMEM for 2 h. Cells were washed three times in DMEM, 10% FCS, supplemented with penicillin and streptomycin. Enriched MAIT cell preparations (1×10^5) were added for an overnight coculture. Cells were then harvested and stained for FACS analysis. For ligand preparation, E. coli wild-type and mutant strains were cultured to stationary phase overnight in Luria broth at 37˚C, washed in PBS, and resuspended in water for osmotic lysis at 4˚C for 6 d. Supernatant was filtered through 0.22 μM and ultra-filtered through a 3-kDa centrifugal filter unit (Amicon; Millipore). The flow-through was finally lyophilized and kept at −20˚C for further use. Fractions are expressed in a.u., with 1 a.u. equivalent to 25 μl bacterial supernatant.

Flow cytometry

Flow cytometry was performed with directly conjugated Abs (BD Pharmingen), according to standard techniques, with analysis on an LSR II flow cytometer (Becton Dickinson). DAPI and 405-nm excitation were used to exclude dead cells. The following Abs, mostly from BD Pharmingen or eBioscience, were used in mice: anti-Vβ6–PE or –FITC (RR4-7), anti-Vβ8–PE or –FITC (F23.1), anti-βTCR–PC5 (H57-597), anti-CD8α–-Alexa Fluor 700 (Ly-2), anti-CD4–PC7 (L3T4), anti-CD69–PE–Texas Red (H1.2F3), anti-CD25–PE–Texas Red (PC61), and anti-MR1 (clone 26.5; Ted Hansen, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO).

Synthesis of 5-A-RU and 5-nitroribitylaminouracil

D-rbitylamine was prepared in 75% yield via a one-step reductive amination from D-ribose followed by purification on ion-exchange resin. Condensation with commercially available 6-chloro-5-nitropyrimidine-2,4(1H,3H)-dione (or 6-chloro-5-nitrouracil) gave 5-nitroribitylaminouracil (5-N-RU) as a yellow powder after purification on an ion-exchange column (45%). Hydrogenolysis of 5-N-RU gave the unstable diaminouracil 5-A-RU.

Synthesis of pteridine variants

Compound A (Cp-A), compound B (Cp-B), compound C (Cp-C), and compound D (Cp-D) were synthesized according to reported procedures (27–29) (Supplemental Fig. 3). The commercially available 2-amino-3-cyano-5-chloromethylpyrazineN-oxide was first converted into 2-amino-4-hydroxy-6-formylpteridine dimethyl acetal in seven steps, yielding Compound A as a light yellow powder (overall yield 9%). Controlled hydrolysis of Cp-A with aqueous formic acid, followed by thorough washing with water, ethanol, and ether gave the 6-formyl pterin (6-FP) derivative (Cp-B) as a beige powder (overall yield 5%). 2-Acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal (Cp-C), which corresponds to the N-protected form of Cp-A, was prepared by a standard acetylation conducted in acetic anhydride at 100˚C for 5 h. After cooling, the precipitate was filtered and washed with cold acetic anhydride, toluene, and ether, followed by thorough washing with water to yield Cp-C as a beige powder (overall yield 2%). Finally, 2-acetylamino-4-hydroxy-6-formylpteridine (Cp-D) was obtained by hydrolysis of Cp-C (overall yield 1%). The overall poor yields were due to the low solubility of these compounds in organic solvents and, thus, the great difficulty in purifying them with classical flash chromatography procedures. The purity of the compounds was established using proton NMR spectra and electrospray ionization mass spectrometry.

Results

MAIT cells are activated by Gram-negative bacteria–derived semipurified fractions

We previously showed that most bacteria and yeast activate MAIT cells (8). Moreover, it was shown recently that riboflavin precursor derivatives from Gram-positive bacteria and synthetic compounds bind to MR1 to activate Jurkat cells transduced with human MAIT cell TCRs (9, 10). The putative heterogeneity of the MAIT cell TCR repertoire and the disparity of human MAIT cell reactivity according to bacteria (11) prompted us to analyze ligand formation in a Gram-negative bacterium (E. coli). We first developed a method to reproducibly generate a semipurified soluble bacterial (SPB) fraction with potent MAIT cell stimulatory activity (Materials and Methods). Serial dilutions of the SPB fraction were applied onto the murine fibroblasts cells (WT3-m and WT3) (1). Enriched MAIT cells from iVα19 Cα$^{-/-}$-Tg (iVα19-Tg) mice were cocultured overnight. Activation was measured by expression of both CD69 and CD25 at the surface of MAIT cells (Fig. 1). The E. coli SPB fraction specifically activated MAIT cells in a dose- and MR1-dependent manner because MAIT cells upregulated CD69 and CD25 only after stimulation by WT3-m plus ligand. Between 40 and 60% of Vβ6/8$^+$ DNCD8^{lo} (MAIT cells) T cells were activated, suggesting a great diversity in the TCRβ repertoire because the MAIT cells of these iVα19-Tg mice display a naive (CD44^{low}/CD62^{high}) phenotype (6) (C. Soudais and O. Lantz, unpublished observations). The small activation observed with parental WT3 cells at high doses of ligand is probably due to the endogenous expression of MR1 (1). Thus, the SPB fraction that we produced from Gram-negative bacteria activates MAIT cells in an MR1-dependent manner.
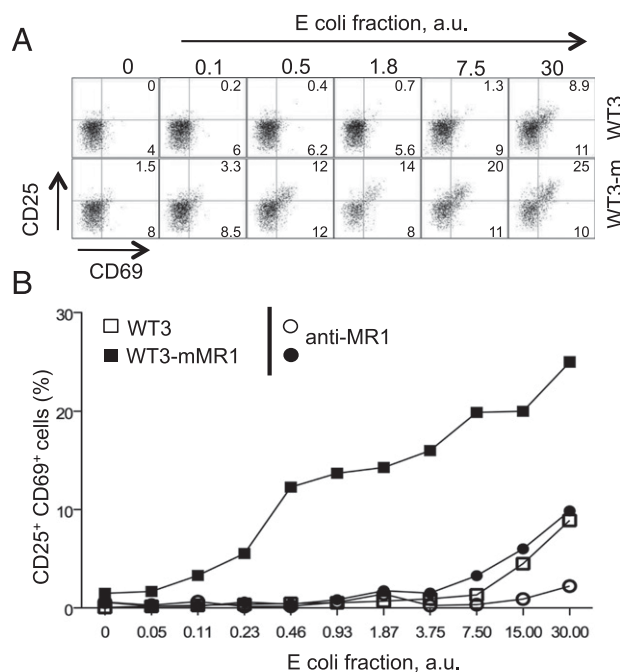

FIGURE 1. A semipurified fraction from a Gram-negative bacterium activates MAIT cells. (**A**) Activation (coexpression of CD25 and CD69) of MAIT cells from iVα19-Tg mice after overnight coculture with serial dilution of E. coli semipurified fraction and WT3-m cells and WT3 cells. (**B**) Activation of MAIT cells according to dilution of the semipurified fraction, with or without anti-MR1 Ab (26.5, 10 μg/ml). Because the plateau of the dose/activation curves varies by up to 2-fold from one experiment to another, with the maximum ranging from 25 to 50%, we chose to show a representative experiment. Data are representative of seven experiments.

5-A-RU found in Gram-negative bacteria is sufficient and necessary for MAIT cell activation

In Gram-positive bacteria, the origin of the MAIT cell ligand(s) was recently assigned to metabolites derived from intermediates of the riboflavin biosynthesis pathway (9). Mutations of the genes in the *rib* operon were made in Gram-positive bacteria (10). To ascertain the origin of the MAIT cell ligand in Gram-negative bacteria, we generated individual mutants for the different steps of the riboflavin biosynthesis pathway. Starting with the available auxotrophic *ribC* mutant obtained from the *E. coli* Genetic Stock Center, we engineered mutants for all of the *rib* genes of the pathway (*Materials and Methods*, Fig. 2A). To measure the ability of the different bacterial mutants to activate MAIT cells, we cultured WT3-m cells with mutant and parental *E. coli* strains at different multiplicities of infection (MOIs). After 2 h of incubation, cells were washed extensively to remove bacteria before overnight coculture with *i*Vα19-Tg MAIT cells in complete media with antibiotics. Activation of MAIT cells was measured as above. As shown previously, *E. coli* activated MAIT cells in an MOI-dependent manner (8). *ribA* and *ribD E. coli* mutants did not activate MAIT cells, whereas *ribB* and *ribE* did (Fig. 2B, 2C). These results are in agreement with the observations made in the Gram-positive bacteria *rib* mutants (10). We then prepared SPB fractions from the same set of bacterial mutants (*Materials and Methods*). When tested in vitro on WT3-m fibroblasts, these SPB fractions recapitulated the observations made with whole bacteria (Fig. 2D). SPB fractions from *ribA* and *ribD* mutant *E. coli* did not activate MAIT cells, whereas SPB fractions from *ribE* mutant and parental *E. coli* did. Taken together, these results suggest that the 5-amino-6-ribitylamino-2,4-(1*H*, 3*H*)-pyrimidinedione (5-A-RU) that is located upstream from the RibE enzyme in the riboflavin pathway is a key intermediate in the generation of MAIT cell ligand in Gram-negative bacteria.

5-A-RU can react with small molecules to activate MAIT cells

Small metabolic molecules present in eukaryotic and bacterial cells, such as glyoxal (Glyx) or methylglyoxal (MeG), can nonenzymatically react with the amine function of 5-A-RU (30) to generate pyrimidine adducts that are potent activators of MAIT cells (10). To determine whether similar processes were occurring with mouse MAIT cells, we synthesized 5-A-RU. To assess the specificity of the amine function of 5-A-RU, we also tested 5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (5-N-RU) (Fig. 3A). In addition to Glyx and MeG, we tested dihydroxyacetone (DHA) (Fig. 3A). DHA is produced as a metabolic intermediate during glycolysis, and intracellular conversion to MeG can occur (31). Thus, we extemporaneously mixed 5-A-RU or 5-N-RU with Glyx, MeG, or DHA. Serial dilutions were put on WT3-m fibroblasts. To assess a putative role for the TCRβ repertoire, we compared MAIT cells with a diverse TCRβ repertoire (*i*Vα19 Tg) (STg) and with a quasi-monoclonal repertoire (*i*Vα19 Vβ6) (DTg). The SPB fraction from *E. coli* was used as a positive control. The nitro derivative 5-N-RU did not activate either STg or DTg MAIT cells on its own or in combination with MeG or DHA, confirming the necessity of the secondary amine function of 5-A-RU to generate the active pyrimidine adducts (Fig. 3B, 3C, Glyx data not shown). The greatest MAIT cell activation was obtained when 5-A-RU was extemporaneously mixed with MeG (~8-fold more efficient that the SPB fraction). Notably, 5-A-RU plus DHA or 5-A-RU plus Glyx were less potent than 5-A-RU plus MeG, but they still reached equivalent levels to the SPB fraction. 5-A-RU alone also activated MAIT cells, probably through its ability to react with carbonyl compounds of the metabolic pathway in eukaryotic cells. No differences in the pattern of reactivity to chemicals were observed using STg or DTg MAIT cells (Fig. 3B, 3C). Because MAIT cells preferentially expressed Vβ6 and Vβ8
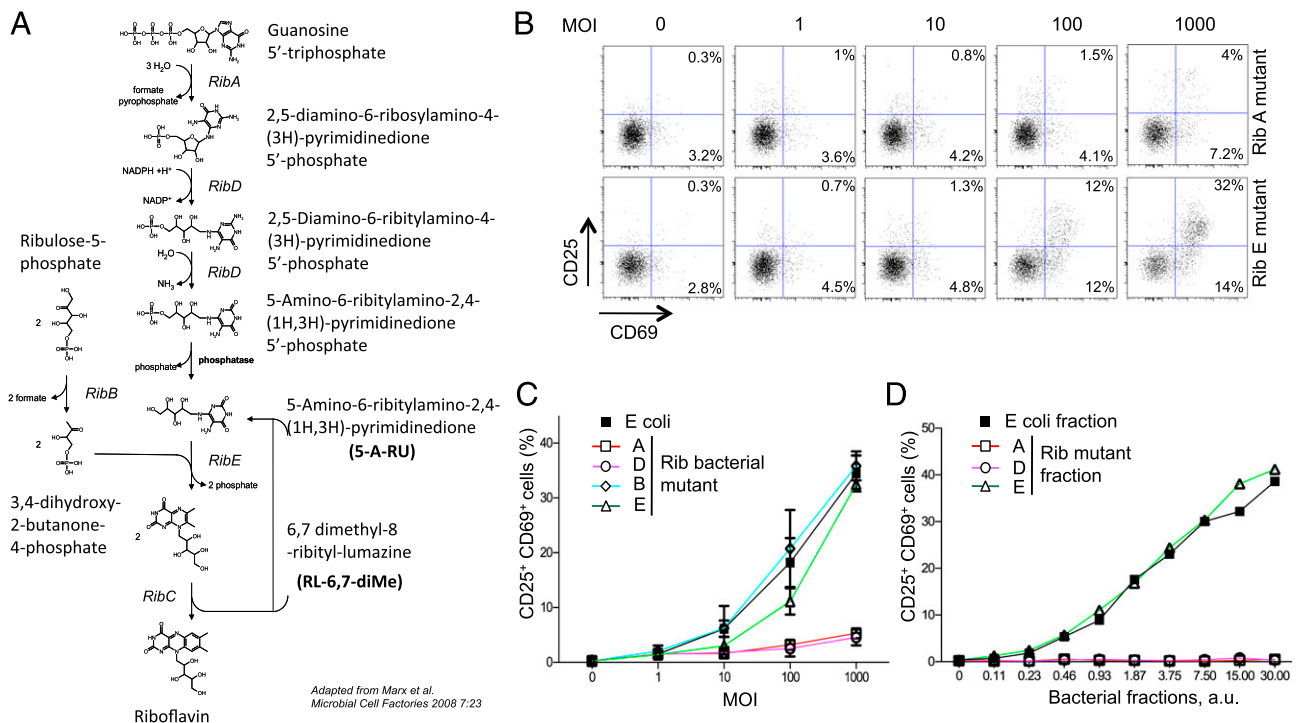
FIGURE 2. Synthesis of 5-A-RU is necessary and sufficient to activate MAIT cells. (**A**) Riboflavin synthesis pathway in *E. coli* (adapted from Ref. 36). Rib genes are in italics, and abbreviated riboflavin intermediates names are in bold. (**B**) Activation of MAIT cells from *i*Vα19-Tg mice after coculture with WT3-m fibroblasts and serial MOI of *ribA* and *ribE* mutant bacteria. (**C**) Activation of MAIT cells by the different *rib* mutant and parental *E. coli* strains. Results are pooled from five separate experiments (mean ± SD). (**D**) Activation of Tg MAIT cells by semipurified fractions prepared from *rib* mutants. Data are representative of four experiments.
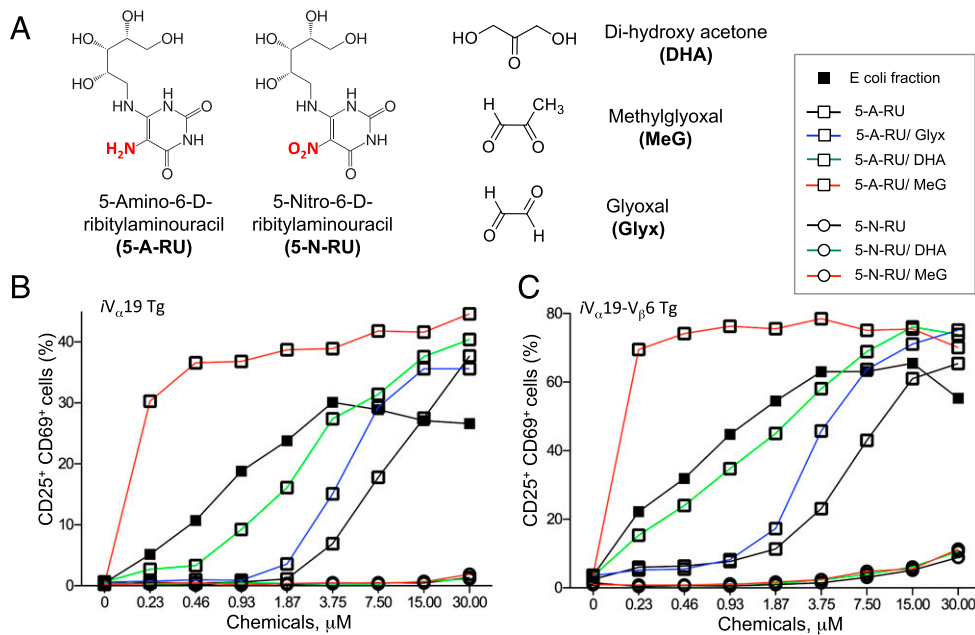
FIGURE 3. 5-A-RU reacts with small metabolites to activate murine MAIT cells from STg (iVα19) and DTg (iVα19Vβ6) animals. (**A**) Chemical structure of the different compounds used. (**B**) Activation of MAIT cells from iVα19-Tg mice after coculture of WT3-m cells incubated with the indicated compounds (Glyx, DHA, or MeG). Data are representative of three independent experiments. (**C**) Same experiment as in (B), using cells from iVα19Vβ6-DTg mice as responding cells. Data are representative of three independent experiments.

segments, we further analyzed the activation of MAIT cells according to Vβ6 or Vβ8 expression in STg MAIT cells. The pattern of reactivity was similar for the two sets of MAIT cells, suggesting a homogeneous reactivity toward the different chemicals tested (Supplemental Fig. 1A–C). Collectively, these data demonstrate that 5-A-RU can react extemporaneously with Glyx and MeG to generate pyrimidine adducts that activate mouse MAIT cells. Thus, the TCRβ-chain does not seem to greatly affect ligand recognition, because the same reactivity was observed with mouse MAIT cells in the context of a polyclonal (STg) or quasi-monoclonal (DTg) repertoire.

In vivo validation of MAIT cell activation

Although there is an in vitro and in vivo correlation between MAIT cell activation and riboflavin-sufficient or -deficient bacteria, other pathways could be involved in vivo. We directly injected the ligand prepared from riboflavin-sufficient or -deficient bacteria or the synthetic compounds into STg animals. Spleens and peritoneal lavage fluid were recovered, and MAIT cell activation was measured. MAIT cells were activated in vivo by the SPB fraction from the parental *E. coli* and from the *ribE* mutant, whereas no activation was observed in the presence of the *ribA* and *ribD* SPB fractions (Fig. 4A). Interestingly, in vivo, MAIT cells were not activated by 5-A-RU alone (Fig. 4B). This is probably due to the instability of 5-A-RU and/or its inability to reach the relevant cellular compartment to react with small metabolites in situ to be loaded onto MR1 (32). In contrast, when 5-A-RU and MeG were mixed extemporaneously before in vivo injection, MAIT cells were activated (Fig. 4B). Altogether, these data show for the first time, to our knowledge, the in vivo activation of MAIT cells by both their specific synthetic and semipurified bacterial ligands. They also demonstrate that in vivo, in a Tg mouse model, MAIT cells are activated by Ags originating from riboflavin-sufficient bacteria but not from riboflavin-deficient bacteria. Moreover, we also confirm that 5-A-RU and MeG can react nonenzymatically to give rise to MAIT cell–activating compounds that can be loaded onto MR1.

Pterin variants increase MR1 surface expression without activating MAIT cells

The above data indicate that the MAIT cell–activating compound(s) present in the SPB fractions is related to the riboflavin biosynthesis pathway and recruits a large MAIT cell repertoire.
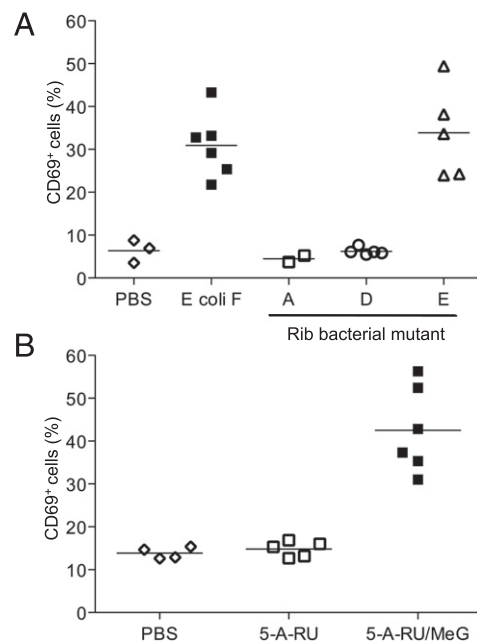
FIGURE 4. Semipurified bacterial fraction and riboflavin precursor derivatives activate MAIT cells in vivo. (**A**) In vivo activation of MAIT cells in iVα19 Cα$^{-/-}$-Tg mice after i.p. injection of 100 a.u. of semipurified fractions prepared from *E. coli* and the indicated Rib mutants. (**B**) Same as (A), after i.p. injection of 100 nmol of the indicated synthetic compounds. Analysis was performed on gated Vβ6/8$^+$ DNCD8^{low} T cells from spleen, 18 h after injection.

However, this compound is not completely identified. One way to indirectly determine the relationships between the SPB fraction and the 5-A-RU–based ligands is to determine their relative affinity for MR1 through competition-binding experiments. To generate nonactivating MAIT cell competitors, we manipulated 6-FP (Cp-B). Because 6-FP can form a covalent Schiff base bond, through its formyl group, with the Lys[43] residue of MR1, we engineered variants of 6-FP by blocking the formyl (Cp-A) or the amine (Cp-D) group of the molecule or both (Cp-C) to prevent the modified molecules from reacting with Lys[43] (9) (see *Materials and Methods*, Fig. 5A). We first examined the ability of these different compounds to increase MR1 surface expression. Serial dilutions of each were incubated for 2 h with WT3-m cells that were then labeled with an anti-MR1 Ab (Fig. 5A, 5B). Cp-C (protected on both sides) and Cp-D (protected on the amine side) greatly increased MR1 expression in a dose-dependent manner (Fig. 5A, 5B). Cp-A, protected on the formyl side, did not increase MR1 expression, whereas Cp-B (6-FP) elevated MR1 expression to a lower extent than did Cp-C and Cp-D. Strikingly, the SPB fraction from *E. coli,* like 5-A-RU plus MeG, did not increase MR1 expression on the surface of WT3-m cells (Fig. 5B, data not shown). Consistent with previous data, 6-FP, as well as the new 6-FP variants, did not activate MAIT cells, making them potential inhibitors of MAIT cell activation (Fig. 5C).

New inhibitors of MAIT cell activation

To test the capacity of these new 6-FP variants to inhibit MAIT cell ligand loading onto MR1 and MAIT cell activation, we set up competition experiments in which we measured both MR1 expression and MAIT cell activation. Serial dilutions of the different compounds were incubated for 2 h with the WT3-m cells before adding serial dilutions of the SPB fraction and MAIT cells. Cp-C, which is blocked on both sides of the 6-FP molecule, prevented activation by the *E. coli* SPB fraction in a dose-dependent manner (Fig. 6B). This result demonstrates that Cp-C has a greater affinity for the MR1 pocket, although Cp-C is not able to form a covalent bond with the Lys[43] residue. Cp-A, which is blocked on the formyl group, and, thus, is unable to form a Schiff base, did not prevent binding of the SPB fraction (Fig. 6A). Both Cp-B and Cp-D prevented the SPB fraction from binding to MR1, but they were not as efficient as Cp-C at increasing MR1 surface expression (Supplemental Fig. 1D, 1E). Altogether, these results show the differential effects of the 6-FP variants. Although Cp-C is unable to covalently bind to MR1, it stabilizes MR1 at the cell surface and appears to be a potent inhibitor of MAIT cell activation.

The semipurified fraction has the same affinity for MR1 as do synthetic compounds

To indirectly assess the relative avidity of the SPB fraction and the synthetic compounds (5-A-RU plus MeG), we performed a competition experiment with Cp-C. Because neither the chemical nature nor the abundance of the MAIT cell–activating compound in the SPB fractions is known, we first performed a titration of the MAIT cell–activating ability of the SPB fraction in comparison with 5-A-RU plus MeG. To perform our competition experiment, we chose to test the amount of SPB fraction and synthetic ligand leading to the same level of MAIT cell activation (Fig. 6C). Serial dilutions of Cp-C were incubated with WT3-m cells before adding
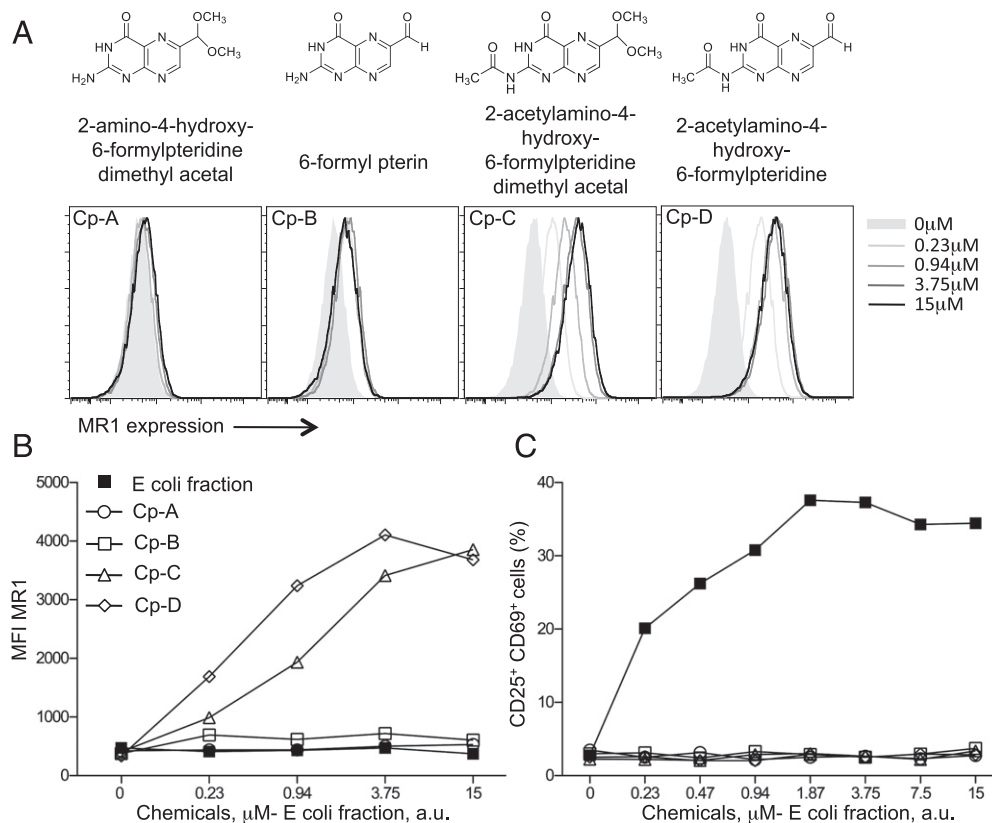

FIGURE 5. Pterin variants increase surface MR1 expression without activating MAIT cells. (**A**) Chemical compounds and examples of MR1 staining of WT3-m cells after incubation with the indicated concentrations of Cp-A, Cp-B, Cp-C, and Cp-D. (**B**) MR1 expression (MFI) by WT-3-m cells after incubation with the indicated compounds or *E. coli* semipurified fraction. Data shown are representative of three independent experiments. (**C**) Activation of MAIT cells from *iVα19-*Tg mice after coculture with WT3-m cells in the presence of the indicated compounds or *E. coli* semipurified fraction. Data shown are representative of three independent experiments.
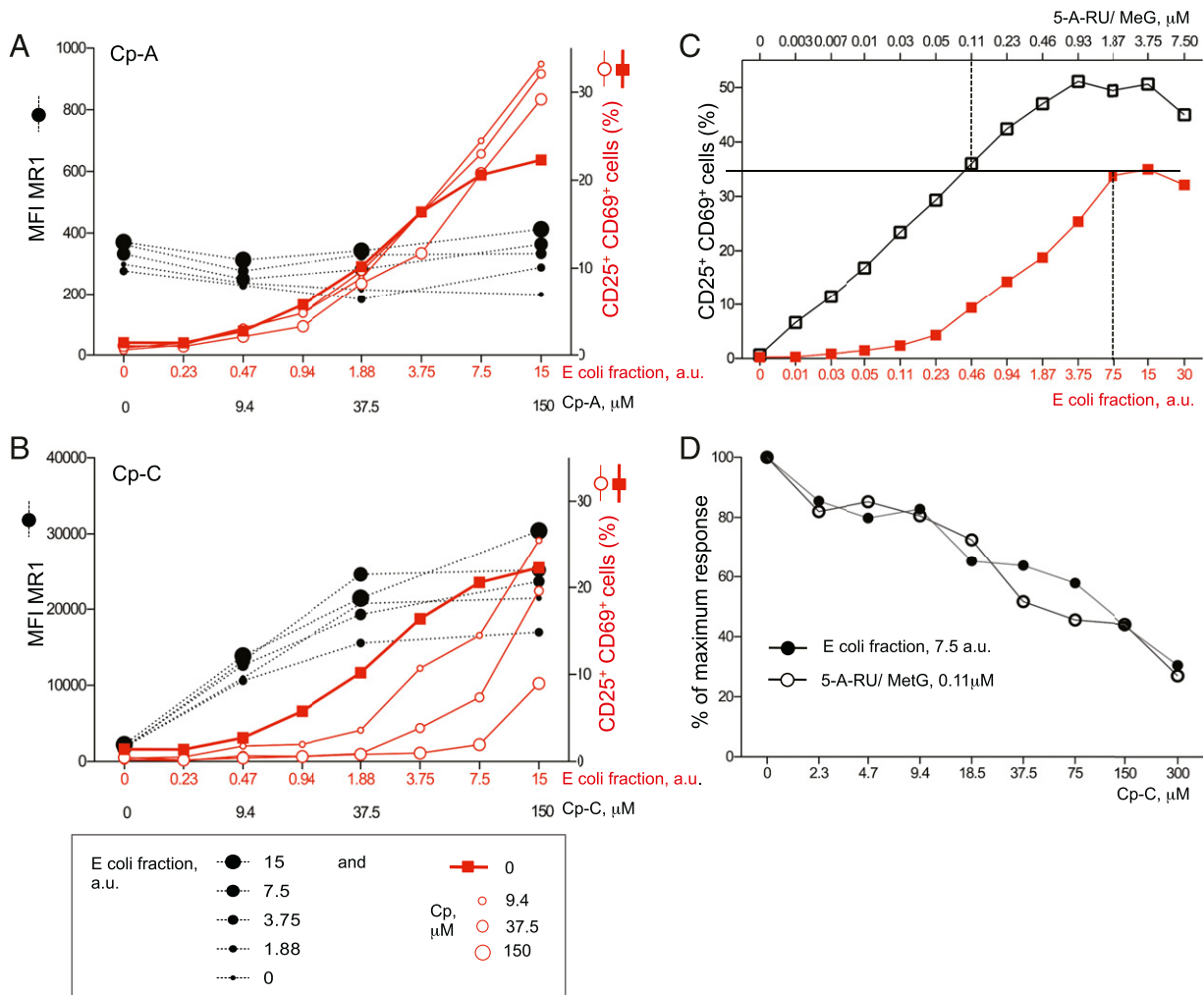
FIGURE 6. The MAIT cell–activating compound found in the SPB fraction and 5-A-RU plus MeG have the same avidity for MR1. Serial dilutions of Cp-A (**A**) or Cp-C (**B**) were incubated with WT3-m cells before adding the indicated dilution of *E. coli* semipurified fraction. MAIT cells from *i*Vα19-Tg mice were then added, and activation was assessed after overnight culture (red curves). MR1 expression by WT3-m cells is plotted (black curves) on the same graph. Data shown are representative of three independent experiments. (**C**) Comparative MAIT cell activation with serial dilutions of *E. coli* semipurified fraction and extemporaneously mixed 5-A-RU plus MeG; 35% MAIT cell activation is reached with 7.5 a.u. of *E. coli* semipurified fraction or 0.11 μM of 5-A-RU plus MeG (dashed lines). (**D**) Serial dilutions of Cp-C were preincubated with 7.5 a.u. of *E. coli* fraction or 0.11 μM of 5-A-RU plus MeG; MAIT cell activation was assessed by CD25 CD69 coexpression, and the results are expressed as the percentage of the maximum response.

an optimal dose of SPB fraction or the synthetic mixture 5-A-RU plus MeG (Fig. 6D). The two inhibition curves were identical, indicating that the biologically active molecular species present in the *E. coli* SPB fraction and in adducts generated from the synthetic compounds have the same avidity for MR1. This competition experiment demonstrates that the biologically active compound that is present in the SPB fraction from *E. coli* is highly similar to adducts formed by the nonenzymatic reaction of 5-A-RU with MeG.

Discussion

So far, the characterization of the bacterial MAIT cell ligand has been achieved using Jurkat cells transduced with human TCRs and, most of the time, Gram-positive bacteria. In this study, using the common intestinal Gram-negative bacteria *E. coli*, we determined the origin of the microbial compound(s) activating mouse MAIT cells. The study of SPB fractions from *rib* mutant bacteria confirmed the major role of 5-A-RU as a precursor for MAIT cell ligand. Moreover, MAIT cell activation by a bacterial or synthetic ligand was evidenced both in vitro and in vivo using two mouse models. Through competition experiments, we showed that an

SPB fraction displayed the same avidity for MR1 as did the most active synthetic MAIT cell ligand, indicating limited heterogeneity of the MAIT cell ligands.

Other than the MR1-restricted bacterial reactivity of murine MAIT cells, the data available on the nature of the ligand activating mouse MAIT cells are limited. In one report (33), murine MR1 tetramers loaded with reduced 6-(hydroxymethyl)-8-D-ribityllu-mazine (rRL-6)-CH$_2$OH (Tet$^+$) were used to stain spleen cells from *i*Vα19-Tg mice (5). Less than 20% of the Vβ6/8$^+$ T cells in the double-negative (DN) or CD4 compartments were stained with this reagent (33). The proportion of Tet$^+$ T cells was still lower for the CD8$^+$ T cells or the Vβ6/8$^-$ T cells. This suggests that either many T cells found in these *i*Vα19-Tg mice are not MR1 restricted or this MR1 tetramer labels only a subset of MAIT cells. Using another *i*Vα19-Tg mouse line, 40–60% of Vβ6/8$^+$ DNCD8^{low} MAIT cells were activated by the SPB fraction or the synthetic ligand (5-A-RU plus MeG) (Fig. 3B, data not shown). Moreover, 60% of the DN T cells found in *i*Vα19 Vβ6–Tg mice were activated by both the SPB fraction and the synthetic ligand (Fig. 3C). In *i*Vα19-Tg mice, the discrepancy between the proportion of T cells labeled with the MR1 tetramer and those acti-

vated by the MAIT cell ligands could be related to differences in the Tg strains studied, the exact tetramer specificity, or the diversity of ligands present in the bacterial extract but not in the tetramer.

The first hypothesis is difficult to address in the absence of a side-by-side comparison of the two Tg lines with both assays. However, because >90% of the DN or CD8 T cells found in the iVα19-Tg lines that we studied display a naive (CD44^{low}/CD62L^{high}) phenotype (6) (Y. Cui and O. Lantz, unpublished observations), their TCRβ repertoire is probably very wide. Therefore, it is probable that TCRβ does not play an important role in MAIT cell specificity: it just has to be permissive. This is consistent with the structural data (34) and with our results showing that a significant (>10%) proportion of Vβ6/8$^+$ T cells found in iVα19C$\alpha^{-/-}$Tap$^{-/-}$Ii$^{-/-}$ mice on a MR1$^{-/-}$ background is activated by bacteria (8) (E. Martin and O. Lantz, unpublished observations). This latter result indicates that the TCRβ-chain does not have to be selected on MR1 to allow bacterial reactivity. Contrasting with the partial labeling of iVα19-Tg T cells by MR1 tetramer loaded with rRL-6-CH$_2$OH, 100% of adult human MAIT cells were stained with the same reagent (33). This discrepancy could be related to a higher expansion of human MAIT cells in comparison with MR1-restricted T cells of iVα19-Tg mice. Only bacteria-specific Vα7.2$^+$CD161^{high} T cells expressing the iVα7.2 TCRα-chain would expand after birth in contact with the commensal, explaining their labeling by the tetramer. In mice, the expression of the iVα19 TCRα-chain is enforced but, as mentioned, the repertoire is highly diverse.

In the second hypothesis, the absence of labeling with the MR1 tetramer could be related to the use of a mutated MR1 (K43A). Indeed, this mutation prevents the formation of a Schiff base between the amine of the MR1 lysine residue and the aldehyde of the pterin moiety to allow loading of rRL-6 during preparation of the tetramer. This would modify the specificity of the tetramer. Native mouse MR1 tetramer loaded with the unstable adduct 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil or 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil may stain a much higher proportion of T cells in iVα19-Tg mice (10). The third hypothesis of bacterial ligand heterogeneity is unlikely, because the synthetic ligand (5-A-RU plus MeG) activated a higher fraction of T cells than did the SPB fraction (Fig. 6B), arguing for limited heterogeneity of the bacterial ligand.

Our data from murine MAIT cells and Gram-negative bacteria mutated for the different enzymes of the riboflavin biosynthesis pathway confirm the key role of the riboflavin precursor, 5-A-RU. We observed that synthetic 5-A-RU induced MAIT cell activation when incubated with MR1-overexpressing cells. The addition of Glyx and MeG greatly increased this activation (Fig. 3), consistent with previous results. Indeed, Glyx and MeG react nonenzymatically with 5-A-RU to generate pyrimidine adducts that bind MR1 and activate MAIT cells (10). It is probable that endogenous MeG from the intermediary metabolism in the APCs reacted with the exogenous 5-A-RU to generate the pyrimidine adducts. In the current study, we also show that DHA increased 5-A-RU–mediated MAIT cell activation (Fig. 3B, 3C). This can either be explained by the formation of adducts after condensation of the amine function of 5-A-RU on the ketone function of DHA at room temperature or by the conversion of DHA into MeG. This conversion is known to occur in eukaryotic cells because DHA may feed into the intermediary metabolism. When added exogenously, MeG reacts with 5-A-RU either in the medium or in the cells. Where and how exactly the pyrimidine adducts are loaded onto MR1 in the cells remain to be determined.

Because bacteria mutated for the two last enzymes of the riboflavin biosynthesis pathway did not lose the capacity to activate MAIT cells, the RL-6,7-diMe (Cp-1) and its hydroxyl derivative RL-6-Me-7-OH (Cp-2), which had originally been proposed as MAIT cell ligands (9), are probably not involved in MAIT cell activation by Gram-negative bacteria. Yet, we synthesized these compounds, as well as the most active one, rRL-6-CH$_2$OH (Cp-3), also proposed in that report, and tested their capacity to activate mouse MAIT cells. Neither Cp-1 nor Cp-2 activated T cells from STg mice, whereas T cells from DTg mice were activated by Cp-1 (Supplemental Fig. 2). The absence of activation of polyclonal MAIT cells by Cp-1 and Cp-2 is consistent with the data obtained using mutated bacteria. The activation by Cp-1 of the quasi-monoclonal T cells from the DTg mice, although less than Cp-3, could be related to a stabilization of MR1 at the cell membrane induced by Cp-1 and some reactivity of the DTg T cells toward the MR1 molecule itself. Indeed, the Vβ6-Tg TCRβ-chain was chosen from a T–T hybridoma that reacts against MR1-overexpressing cells without the addition of bacterial ligand (C. Soudais and O. Lantz, unpublished observations). Both STg and DTg T cells were activated by Cp-3; however, only a small quantity of Cp-3 was obtained in the final crude product. It is likely that the MAIT cell activation that we observed with this compound is related to contaminants, including 5-A-RU (the precursor) or pyrimidine adducts, as suggested by other investigators (10).

Lewinsohn and colleagues (11) suggested some heterogeneity of the bacterial ligands activating MAIT cells. Although we confirmed that 5-A-RU originating from the bacteria is required for MAIT cell activation, we studied how the biological activity found in the SPB fraction relates to the 5-A-RU–derived pyrimidine adducts. For that purpose, we first synthesized 6-FP analogs that would bind MR1 without activating MAIT cells, because they lack the ribityl moiety that interacts with the tip of the CDR3α of the MAIT cell TCR (12). In addition to 6-FP (Cp-B) and acetyl-6-FP (Cp-D, in which the amine is blocked but the formyl group is free) already described (10), we synthesized two new compounds: Cp-A (the formyl group is blocked, preventing the formation of a Schiff base with Lys^{43} of MR1) and Cp-C (both the amine and formyl groups are blocked). As described previously (34), 6-FP and Cp-D increased MR1 expression without activating MAIT cells (Supplemental Fig. 1D, 1E). Consistent with the blockage of the formyl group, Cp-A did not modify MR1 expression. Although its formyl group is blocked, Cp-C was the best at upregulating MR1 expression without activating MAIT cells (Fig. 6B). It is likely that the acetyl group blocking the amine of the pterin in Cp-C and Cp-D stabilized the interaction of these compounds with MR1, leading to its upregulation (10, 16).

Cp-C efficiently inhibited MAIT cell activation by the SPB fraction and the synthetic (5-A-RU plus MeG) ligand. The inhibition curves were identical, suggesting that the avidity for MR1 of the bacterial compound(s) and synthetic ligands were similar. Together with the results using Rib-mutated bacteria, this suggests that the Gram-negative bacteria–derived MAIT cell–activating ligand present in the SPB fraction is the result of the complementation of 5-A-RU with MeG. Because we used living MR1-overexpressing cells to assess MAIT cell activation, and because both 5-A-RU and the pyrimidine adducts are unstable, it is difficult to determine whether MeG is provided by the bacteria or the eukaryotic cells.

As for a putative heterogeneity of MAIT cell TCR reactivity suggested by TCR repertoire analysis of human Vα7.2$^+$ CD8α^+ T cells (11), we do not find any evidence in mice. Indeed, the naive T cells found in iVα19-Tg mice, which harbor a diverse

repertoire, displayed a very homogeneous 5-A-RU–dependent reactivity. Altogether, our data fit with a very limited diversity of ligands, which would stem from 5-A-RU reacting with either MeG or Glyx. However, because the F′ pocket of MR1 is empty in the structural data (17, 34, 35), additional modulation of MAIT cell TCR binding or MR1 trafficking could be related to endogenous or bacterial compounds filling this pocket.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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