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Cytosolic delivery of bacterial metabolites by riboflavin transporters promotes MR1 antigen presentation and MAIT cell recognition

Sebastian Cruz-Gomez[1], Hui Jing Lim[1], Brunda Nijagal[2], Yuting Yang[1,3], Nicole Lau[1], Jeffrey YW Mak[4,5], David P Fairlie[4,5], Hayley Newton[1], Mariolina Salio[6], Jose A Villadangos[1,7] & Hamish EG McWilliam[1]

1 Department of Microbiology and Immunology, The University of Melbourne, The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia
2 Metabolomics Australia, Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne, Melbourne, VIC, Australia
3 School of Medicine, Tsinghua University, Beijing, China
4 Centre for Chemistry and Drug Discovery, Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia
5 Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Queensland, Brisbane, QLD, Australia
6 Nuffield Department of Medicine, University of Oxford, Oxford, UK
7 Department of Biochemistry and Molecular Biology, The University of Melbourne, Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia

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Correspondence
Hamish EG McWilliam and Jose A Villadangos, Department of Microbiology and Immunology, The University of Melbourne, The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC 3000, Australia.
E-mail: hamish.mcwilliam@unimelb.edu.au and j.villadangos@unimelb.edu.au

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Abstract

Major histocompatibility complex class I-related protein 1 (MR1) presents microbial Vitamin B-related metabolite antigens (VitBAg) at the cell surface to activate mucosal-associated invariant T (MAIT) cells. Precisely how antigen-presenting cells capture these MR1 ligands is not known. Here, we show that the most effective route for presentation of bacterial VitBAg involves passage through the cytosol. Consistent with structural similarities with riboflavin, we find that VitBAg presentation is inhibited by riboflavin. We further show that riboflavin carriers transport VitBAg into cells and enhance MR1 antigen presentation to MAIT cells. However, elimination of specific riboflavin carriers does not ablate VitBAg presentation, indicating cells possess redundant mechanisms to internalize this family of MR1 ligands. Our findings provide new insights into the intracellular pathway used by VitBAg to bind MR1 molecules and identify potential approaches to boost MR1-mediated MAIT cell responses for therapeutic benefits.

INTRODUCTION

Antigen presentation by Major Histocompatibility Complex (MHC) molecules is critical for immunity. T cells recognize MHC-antigen complexes presented on the surface of cells, which induces their activation and effector functions. Mammals possess multiple types of MHC molecules that present a diverse range of antigens to encompass various foreign molecular components. Classical MHC class I and II present highly variable peptides from the degradation of proteins, while nonclassical molecules such as MHC class I-related protein 1 (MR1) or the members of the CD1 family present less variable metabolite or lipid antigens, respectively.[1–3] Each antigen presentation system requires accessory proteins and cellular processes dedicated to optimize the production, capture, and/or delivery of antigen ligands to the location where they bind their

particular MHC molecule. In the case of the MR1 presentation pathway, these mechanisms remain largely uncharacterized.

The T cells that recognize MR1-presented antigens are known as MR1-restricted T (MR1T) cells, among which the mucosal-associated invariant T (MAIT) cells are the most abundant. MAIT cells express a semiconserved T-cell receptor (TCR) that recognizes small microbial metabolites bound to MR1.[3–7] These metabolite antigens are derived from the synthesis of riboflavin (vitamin B2),[4,5] termed Vitamin B-related Antigens (VitBAg). As riboflavin is synthesized by fungi and most bacteria[8] but not mammals, the intermediate metabolites of its biosynthetic pathway are a molecular signature of these microbes.[5] The most potent VitBAg is 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), a pyrimidine-based molecule consisting of a single ring derived from the spontaneous combination of riboflavin biosynthetic intermediate 5-amino-6-D-ribitylaminouracil (5-A-RU) with the ubiquitous glycolysis metabolite methylglyoxal.[4] MR1 binds 5-OP-RU derived from intracellular sources such as phagocytosed or cytosolic bacteria,[9–12] or from the extracellular milieu.[13–20] However, 5-OP-RU is inherently unstable[21] and rapidly converts to 7-methyl-8-D-ribityllumazine (RL-7-Me), a weak MAIT cell stimulant. Binding to MR1 prevents this conversion and preserves the activity of 5-OP-RU as a MAIT cell agonist. This implies that to optimize their recognition by MAIT cells, MR1-presenting cells must quickly capture and direct these metabolites to the location where MR1 binds ligands. Two locations have been proposed: the Endoplasmic Reticulum (ER) and endosomal compartments.[3] Cells maintain a pool of newly synthesized MR1 molecules within the ER in a ligand-receptive state[11,14,22,23] and our previous work has concluded the ER is the site where MR1 binds VitBAg.[17,24] The route followed by VitBAg to reach the ER and the mechanisms involved in their transport from the extracellular environment or from the lumen of phagosomes harboring bacteria remain unknown. Other studies have concluded that MR1 traffics in a ligand-receptive state to endosomal compartments where it binds VitBAg, a pathway that would circumvent the requirement for VitBAg transport across membranes.[14,22,24,25] Characterization of the intracellular route followed by MR1 ligands and its mechanisms is critical to fully understand the biology of MR1 antigen presentation. This knowledge may also allow the development of new strategies to augment or inhibit VitBAg uptake by MR1 presenting cells for the purpose of enhancing or impairing beneficial and deleterious MAIT cell responses, respectively.[20,22]

Here, we use intracellular bacterial infection models to show that VitBAg produced by intracytosolic bacteria is more efficiently presented by MR1 than VitBAg produced by bacteria residing in phagosomes. We also show that internalization of extracellular VitBAg is unaffected by inhibitors of fluid-phase endocytosis but is outcompeted by riboflavin, indicating VitBAg and riboflavin are captured by similar mechanisms. Indeed, VitBAg capture and presentation to MAIT cells is enhanced by overexpression of riboflavin transporters.

RESULTS

VitBAg is more efficiently presented by MR1 when it occurs in the cytosol of infected cells

First, we investigated to what extent VitBAg presentation benefits from production of its precursor metabolites in the cytosol. We exploited the fact that during intracellular *Salmonella enterica* serovar Typhimurium (STM) infection, bacteria remain predominantly in phagosomes but can also escape this compartment and reside in the cytoplasm of the infected cells.[26,27] We used a modified STM strain that constitutively expresses mCherry and also contains the P*uhpT-gfpova* plasmid, which is used by the bacteria to produce GFP when they sense glucose-6-phosphate in the host cell cytoplasm.[26] HeLa cells incubated with this strain can be differentiated by flow cytometry as uninfected (mCherry$^-$ GFP$^-$); infected and harboring STM only in phagosomes (mCherry$^+$ GFP$^-$); or infected and containing cytosolic STM (mCherry$^+$ GFP$^+$) (Figure 1a, b). MR1 is expressed on the plasma membrane only if it is bound to antigen ligands[14] and at levels proportional to the amount of VitBAg produced and delivered to the ER, the intracellular location where MR1 binds its ligands.[3,14,15,25] Correspondingly, uninfected HeLa cells expressed similar levels of MR1 as cells not incubated with bacteria, whereas infected cells up-regulated MR1 surface expression (Figure 1d). Notably, cells that contained cytosolic bacteria displayed more MR1 than cells that contained bacteria only in phagosomes (Figure 1d).

The number of cells containing cytosolic STM was small, so we also used two STM mutant strains that lack the genes *sopF* or *sifA*, which escape more readily to the cytosol of infected cells.[26,27] The proportion of host cells infected (mCherry$^+$) with WT and the two mutant bacteria was equivalent (Figure 1b, c). Neither of the infection models caused significant host cell death (Supplementary Figure 1a) or altered *Mr1* transcription (Supplementary figure 1b). However, the proportion of cells infected with the *sopF* and *sifA* mutant strains that contained bacteria in the cytosol (mCherry$^+$ GFP$^+$)

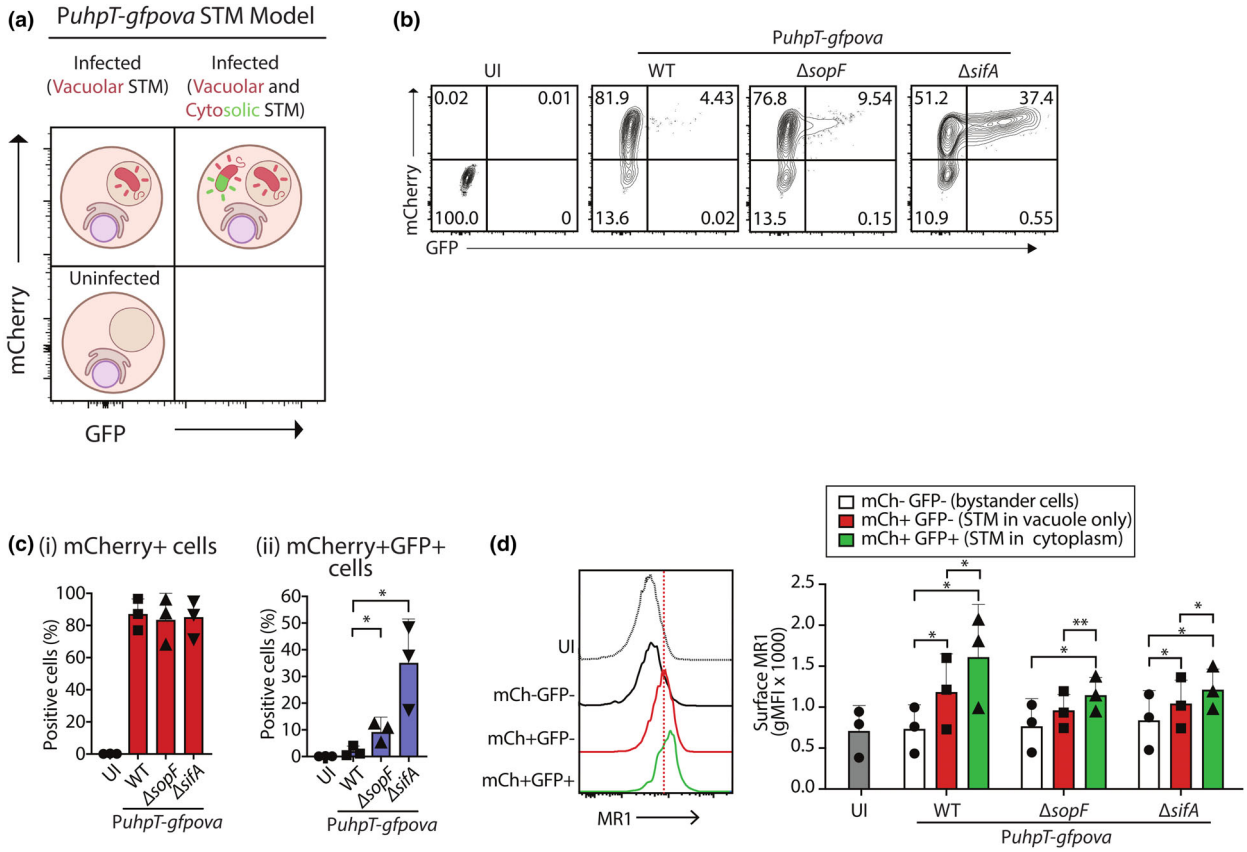

Figure 1. Cytosolic *Salmonella enterica* promotes MR1 presentation. **(a)** Diagram showing the resulting populations after P*uhpT-gfpova* STM infection. **(b)** Representative flow cytometry plot of HeLa cells uninfected (UI), infected with wild-type (WT), or mutants Δ*sopF* or Δ*sifA* of mCherry-expressing *Salmonella enterica* serovar Typhimurium (STM) for 8 h. All STM strains also expressed the cytosolic reporter plasmid encoding P*uhpT-gfpova*. Within the infected conditions, HeLa cells harboring intracellular STM are mCherry+, and those with cytosolic STM are also GFP+, while bystander cells are mCherry-GFP-. **(c)** Rate of intracellular STM infections or cytosolic bacteria for each WT or mutant strain, measured by the percentage of mCherry+ (i) or mCherry+GFP+ cells (ii), respectively. **(d)** The cell surface MR1 of each UI or infected condition from **(a)**, measured by flow cytometry. Each result shown is the mean of three independent experiments. Statistical significance was calculated using a one- **(c)** or two-way ANOVA **(d)** using Tukey or Šídák's multiple comparison test, respectively, comparing each group against each other where $*P < 0.05$ and $**P < 0.01$.

increased two- and ninefold, respectively (Figure 1b, c). As was the case in cells infected with WT STM, cells containing mutant bacteria in their cytosol had higher levels of surface MR1 than cells harboring bacteria only in phagosomes (Figure 1d). It is important to appreciate that, while mutant bacteria escape more readily to the cytosol, this does not imply that cells infected with such bacteria contain more VitBAg available for MR1 binding than cells harboring WT bacteria in their cytosol. The conclusion is that, regardless of how much VitBAg is produced in infected cells, direct generation in the cytosol leads to more efficient delivery of the ligand to MR1-receptive molecules and subsequent MR1 expression on the cell surface than production of the ligand only in phagosomes. This supports the notion that MR1 binds VitBAg in the ER following passage of the ligand through the cytosol.

Extracellular VitBAg presentation does not require endocytosis

VitBAg produced in phagosomes harboring bacteria might follow two routes to access the cytosol. The first could consist of transfer from the phagosomal lumen across the limiting membrane by diffusion or dedicated transporters, or following partial or complete disruption of membrane integrity, a mechanism that enables MHC I cross-presentation of endocytosed protein antigens.[2] The

second route could involve secretion of VitBAg from infected cells to the extracellular environment, from where they may access the cytosol using plasma membrane transporters. Either or both pathways may also be followed by VitBAg produced extracellularly. If transport to the cytosol requires accessing the endosomal route, reducing endosomal uptake might decrease the efficiency of extracellular VitBAg presentation. To test this, we pulsed C1R cells with a nonsaturating dose of the archetypical VitBAg, 5-OP-RU (1 μM) and tested the effect of inhibitors of endocytosis. In these experimental conditions, any reduction in 5-OP-RU uptake would cause a reduction in surface MR1 upregulation. Even though cytochalasin D (CCD) and ethylisopropyl amiloride (EIPA) inhibited uptake of the fluid-phase endocytic tracer, dextran (Figure 2a, c), neither reduced MR1 presentation of 5-OP-RU (Figure 2b, d). This observation suggests that the efficiency of endocytic uptake is not a rate-limiting step for VitBAg presentation.

Riboflavin specifically inhibits MR1 cell surface upregulation by 5-OP-RU

The chemical structure of 5-OP-RU is too polar to cross the plasma membrane effectively by passive diffusion. Its transport from the extracellular medium to the cytosol is more likely mediated by a transporter. No such transporter has been described but given the structural similarity between riboflavin and 5-OP-RU (Supplementary figure 2a), we considered that riboflavin transporters may be involved in 5-OP-RU uptake. We reasoned that if that were the case, riboflavin should inhibit 5-OP-RU uptake and MR1 presentation in a concentration-dependent manner. This was indeed the case (Figure 3a). Thiamine (vitamin B1, Supplementary figure 2a), which does not inhibit riboflavin import,[28] did not impair 5-OP-RU presentation (Figure 3a). In contrast, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), metabolites that are structurally related to riboflavin (Supplementary figure 2a) and can inhibit riboflavin uptake[28] also impaired 5-OP-RU presentation (Figure 3a). Neither of the four compounds affected MHC I expression (Supplementary figure 2b). Riboflavin, FMN, and FAD also reduced the ability of C1R cells to present MR1-5-OP-RU complexes to Jurkat cells expressing a MAIT TCR (Jurkat.MAITΔβ_2m cells), but thiamine did not (Figure 3b).[11] Furthermore, riboflavin inhibited recognition of MR1-5-OP-RU by primary MAIT cells presented by THP-1 cells (Figure 3c), but it did not impair recognition of CD1d- or MHC I-presented antigen by cells expressing NKT and CD8 T-cell receptors, respectively (Supplementary figure 2c, d). The compound Ac-6-FP is a well-characterized MR1 ligand but, unlike 5-OP-RU, it is structurally distinct from riboflavin and not derived from the riboflavin metabolic pathway.[29] Neither of the compounds that inhibited 5-OP-RU presentation by MR1 inhibited Ac-6-FP presentation (Figure 3a). This implies that the inhibitors did not disable the antigen-presentation properties of MR1 *per se*, but that they inhibited 5-OP-RU uptake specifically. These experiments support the hypothesis that VitBAg use the same mechanism of uptake employed by cells to internalize riboflavin.

Next, we used hydrophilic interaction liquid chromatography (HILIC) coupled to mass spectrometry (MS) to directly measure cellular uptake of 5-OP-RU,[21] and its inhibition by pre-incubation with riboflavin. Using electrospray ionization in negative mode, 5-OP-RU and riboflavin have mass-to-charge ratios (m/z) of 329.11,[4] and 375.13, respectively (Figure 3d). The 329.11 molecular species (5-OP-RU) was detected in lysates of cells incubated with 5-OP-RU, but not in lysates of cells

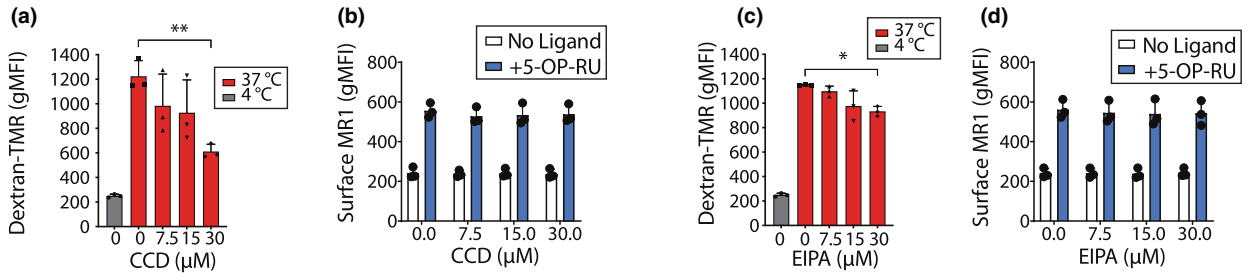

Figure 2. Endocytosis inhibition on MR1-5-OP-RU upregulation. C1R cells were treated with cytochalasin D **(a, b)** at a range of 0 to 30 μM or 5-(N-Ethyl-N-isopropyl)-Amiloride (EIPA) **(c, d)** at a range of 0–30 μM, then incubated with tetramethylrhodamine-labeled dextran 70 kDa (Dextran-TMR) **(a, c)** or 1 μM 5-OP-RU **(b, d)**. The uptake of Dextran-TMR or cell-surface MR1 was detected by flow cytometry. Data presented are the mean of gMFI ± SD of three independent experiments. Statistical significance was calculated using one-way ANOVA using Tukey's multiple comparison test comparing each group against each other where $*P < 0.05$ and $**P < 0.01$.

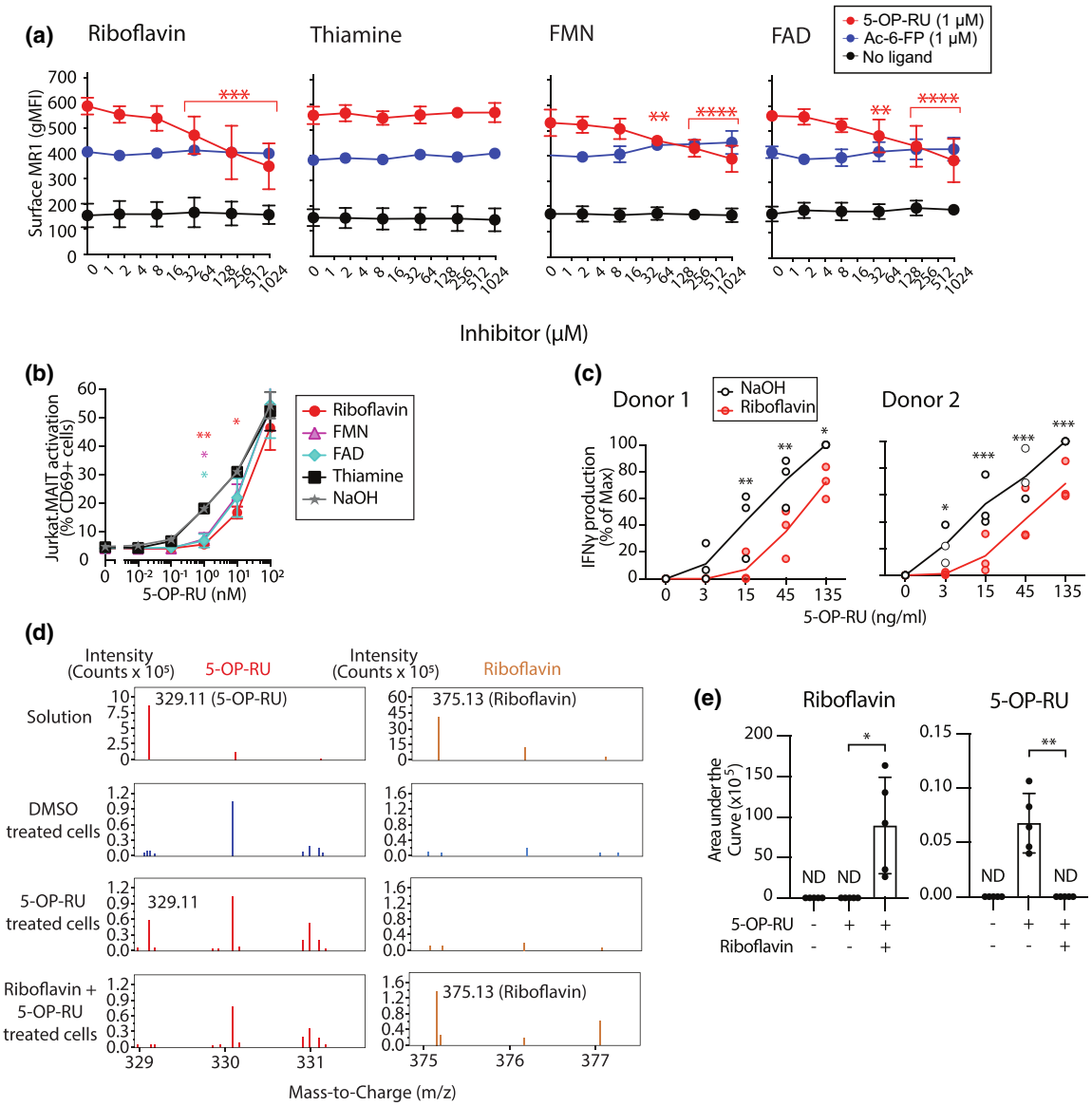

Figure 3. Inhibition of MR1-5-OP-RU presentation by riboflavin. **(a)** C1R cells were pre-incubated for 30 min with titrating amounts of each vitamin or riboflavin derivative and then subjected to a pulse assay with or without 5-OP-RU at 1 μM or Ac-6-FP at 1 μM then surface MR1 was measured. **(b)** C1R cells were pre-incubated for 30 min with riboflavin, FMN, FAD or thiamine at 1 mM or NaOH (Vehicle) at 100 nM and then subjected to a pulse assay with the indicated amounts of 5-OP-RU in the presence of such inhibitors, then cells were co-incubated with Jurkat.MAIT cells for 16 h. Jurkat.MAIT cell activation was evaluated measuring CD69 by flow cytometry. **(c)** THP-1 cells were pre-incubated with 1 mM riboflavin or vehicle (NaOH at a final concentration of 100 nM) and then subjected to a pulse assay with the indicated amounts of 5-OP-RU, then incubated overnight with PBMCs from two healthy donors. MAIT cell activation was assessed by assessing the levels of IFNγ secreted present in the media. **(d)** C1R cells were first incubated with or without riboflavin (1 mM) for 30 min then with or without 5-OP-RU (10 μM) for 1 h, then lysates subjected to HILIC-MS, mass to ratio data is shown. Measurement of riboflavin and 5-OP-RU is shown in **(e)**. Data presented are the means of gMFI ± SD of three independent experiments **(a, b)**, three independent experiments from each of two individual donors **(c)** or 5 biological replicates **(d, e)**. Statistical significance was calculated using one **(e)** or two-way ANOVA **(a, b, c)** using Tukey or Šídák's multiple comparison test, respectively, comparing each group against each other where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

incubated with DMSO vehicle (Figure 3d, e; Supplementary figure 3). Moreover, when we incubated cells with both riboflavin and 5-OP-RU, only the 375.13 molecular species (riboflavin) was detected in lysates, confirming that riboflavin inhibited 5-OP-RU uptake (Figure 3d, e; Supplementary figure 3).

Together, these results show that riboflavin and its derivatives can inhibit the surface display of MR1-5-

OP-RU complexes. The specific inhibition of 5-OP-RU but not Ac-6-FP is consistent with the notion that riboflavin inhibits 5-OP-RU transport into cells without blocking the ability of MR1 to bind and present ligands, suggesting that 5-OP-RU and riboflavin use the same transport mechanisms to gain intracellular access.

Riboflavin transporters are not essential for the uptake of 5-OP-RU

Humans express three riboflavin transporters: SLC52A1, SLC52A2, and SLC52A3.[30] We investigated whether their expression is essential for 5-OP-RU uptake. We deleted these transporters in C1R cells using CRISPR-Cas9 gene editing and two single-guide RNAs (sgRNA) for each (Supplementary table 1). First, we generated clonal populations of knockout (KO) cells lacking only one transporter. We confirmed by DNA sequencing that the selected clones contained frame-shift mutations in the targeted genes (Supplementary table 1). Next, we made double and triple KO by subsequent rounds of gene targeting (Supplementary table 1). As riboflavin is an essential vitamin, we examined the impact of the deletions on cell growth and survival in culture medium containing two concentrations of riboflavin: 1 μM (the concentration in standard media) or 25 nM (the physiological concentration of riboflavin).[31] All mutant cells grew normally in medium containing 1 μM riboflavin, but growth of cells lacking SLC52A2 alone or in combination with one or more of the other transporters showed a 10-fold reduction in cell growth (Supplementary figure 4, note the logarithmic scale). This implies, first, that SLC52A2 has a more prominent role in riboflavin import than SLC52A1 or SLC52A3, at least in C1R cells. Second, our results show that SLC52A2 is required for riboflavin import in C1R cells when the vitamin is present at physiological levels but becomes nonessential at high concentrations of riboflavin, probably because free diffusion or alternative mechanisms of riboflavin uptake compensate for the loss of SLC52A2.

We measured the ability of the mutant cell lines to capture and present MR1 ligands. All upregulated MR1 similarly after incubation with 5-OP-RU (Figure 4a) or Ac-6-FP (Supplementary figure 4b). Further, the cell line that lacked expression of the three transporters was still able to present 5-OP-RU and activate Jurkat.MAITΔβ_2m (Figure 4b). We used HILIC-MS to confirm the effect of SLC52A member deletions on VitBAg uptake. We observed that cells that lacked only SLC52A2 or all three transporters SLC52A1-A3 still captured 5-OP-RU as efficiently as nonmutant cells (Figure 4c). These results imply that SLC52A1–3 are not strictly necessary to import VitBAg into cells or that, like riboflavin, they can be internalized by another pathway or other transporters when SLC52A1–3 have been artificially eliminated.

Overexpression of riboflavin transporters enhances 5-OP-RU uptake and presentation

While the riboflavin transporters are not essential for 5-OP-RU uptake, our finding that riboflavin can inhibit 5-OP-RU uptake suggests that these transporters may still represent a route for antigen uptake and thus a target to modulate MR1 presentation. To test this, we overexpressed each riboflavin transporter in C1R cells fused to a hemagglutinin tag at the carboxy terminus to facilitate detection (Figure 5a; Supplementary figure 5a). We also expressed the folate transporter (SLC19A1) as a negative control (Figure 5a; Supplementary figure 5a). When these cell lines were pulsed with 5-OP-RU, overexpression of SLC52A1 and A2 caused significantly higher MR1 upregulation compared to those transduced with the empty vector (EV) (Figure 5b). Overexpression of SLC52A3 or SLC19A1 had no effect. Presentation of Ac-6-FP was not affected by overexpression of any of the transporters (Figure 5b) and all mutant cell lines expressed similar levels of MHC class I (Supplementary figure 5B). Furthermore, C1R cells overexpressing SLC52A1 and -A2 presented 5-OP-RU and activated Jurkat.MAITΔβ_2m cells to a greater extent than their counterparts transduced with empty vector, or overexpressing SLC52A3 or SLC19A1, did (Figure 5c). Even though SLC52A1 was overexpressed at lower levels than SLC52A2 (Figure 5a; Supplementary figure 5a), it appeared to better promote 5-OP-RU uptake, as indicated by increased detection of MR1-VitBAg complexes on the surface of the C1R cells (Figure 5b) and increased activation of the MAIT TCR on Jurkat cells mediated by these complexes (Figure 5c). These results led us to directly measure 5-OP-RU uptake mediated by this mutant using HILIC-MS. Strikingly, SLC52A1 overexpression did not alter riboflavin uptake, but it increased 5-OP-RU uptake 10-fold (Figure 5d). Excess riboflavin outcompeted 5-OP-RU uptake by both control and SLC52A1-overexpressing C1R cells (Figure 5d), indicating the overexpressed transporter retained its normal function. This result implies that SLC52A1 can transport 5-OP-RU, and that riboflavin can block this process.

Next, we examined the effect of transporter overexpression on presentation of VitBAg produced by bacteria. First, we incubated the control and mutant C1R cell lines with *Escherichia coli* strain DH5α, an extracellular bacterium. While the amount of VitBAg released to the culture medium by the bacteria in these conditions is sufficient for MR1 presentation and

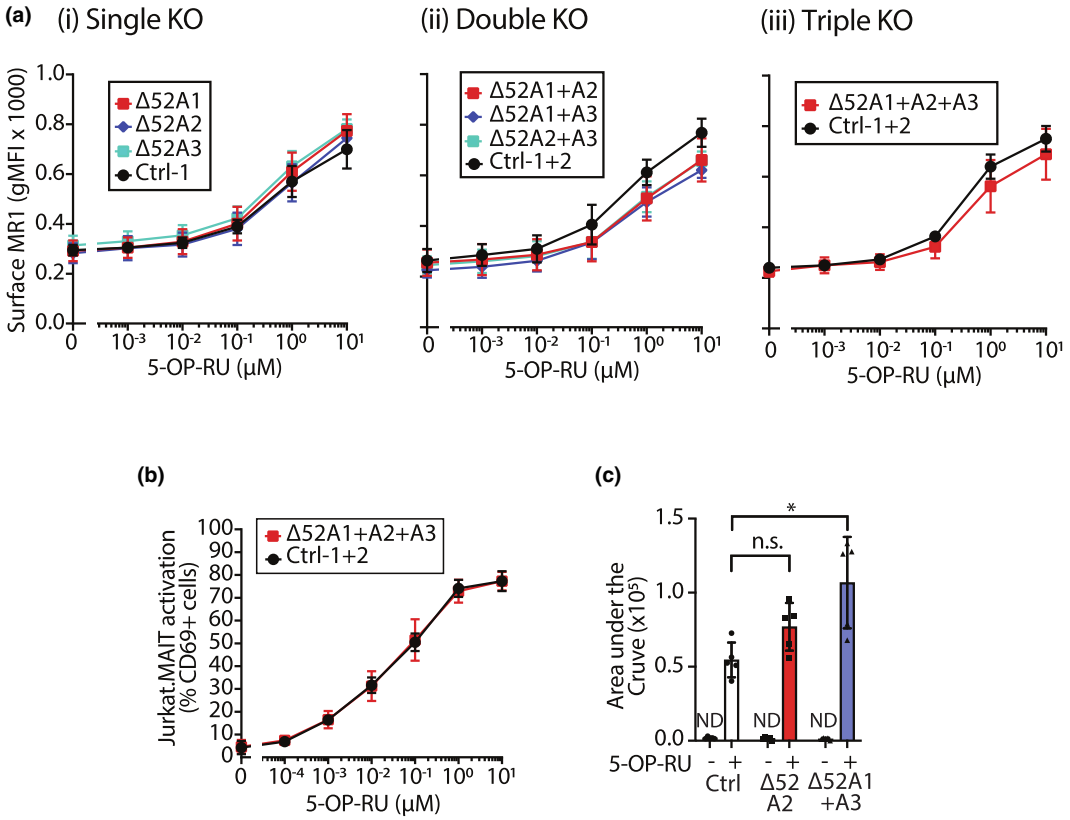

Figure 4. Deletion of riboflavin transporters does not impact MR1-5-OP-RU presentation. **(a)** Single-cell clones of C1Rs knocked out (Δ) for each riboflavin transporter (Δ52A1, Δ52A2, or Δ52A3) individually (i), or in each combination of two knockouts (ii), or all three knockouts (iii), compared to those transduced with one or two control nontargeting sgRNAs (Ctrl-1 or -2). Cells were subjected to a pulse assay with the indicated amounts of 5-OP-RU, then cell-surface MR1 was detected by flow cytometry. **(b)** The triple knockout or Ctrl cell lines from (**a** (iii)) were subjected to a pulse assay with the indicated amounts of 5-OP-RU, then cells were co-incubated with Jurkat.MAIT cells for 16 h. Jurkat.MAIT cell activation was evaluated measuring CD69 by flow cytometry. **(c)** Quantification of 5-OP-RU detected by HILIC-MS in lysates from C1R Ctrl-1, Δ52A2 or Δ52A1 + A3 after treatment with or without 10 μM 5-OP-RU for 1 h. Data represents five replicates per group. Data shown are the mean **(a, b)** ± SD of three independent experiments or five biological replicates **(c)**. Statistical significance was calculated using a one- **(c)** or two-way ANOVA **(a, b)** using Tukey or Šídák's multiple comparison test, respectively, comparing each group against each other where $*P < 0.05$.

MAIT cell recognition by control C1R cells, it is not enough to increase MR1 expression at levels detectable by flow cytometry (Figure 5e).[24] However, cells overexpressing SLC52A1 upregulated MR1 at detectable levels, supporting the notion that they increased bacterial VitBAg uptake. Neither of the other mutant cell lines upregulated MR1 at higher levels than control C1R cells did. All cell lines downregulated MHC class I expression when cocultured with *E. coli* (Supplementary figure 5c), indicating they were similarly exposed to and affected by the bacterium.[32] Finally, we incubated control and mutant C1R cell lines with mCherry-expressing STM and measured MR1 surface expression in infected ($mCherry^+$) or bystander ($mCherry^-$) cells. All infected cells expressed higher levels of MR1 than their bystander counterparts (Figure 5g), but only infected cells overexpressing SLC52A1 expressed higher levels than the control cells (Figure 5g), showing this transporter has the ability to deliver VitBAg produced in endosomes harboring bacteria to the MR1 presentation pathway. Furthermore, the only bystander cells that upregulated MR1 were those overexpressing SLC52A1 (Figure 5g), confirming the transporter could also mediate uptake of VitBAg released to the extracellular medium (Figure 5g). Cells infected with a VitBAg-deficient STM strain (SL1344ΔRibDH)[4] failed to upregulate MR1, so the changes in MR1 expression observed with the WT STM strain were mediated by VitBAg capture and presentation. In conclusion, SLC52A1 overexpression enabled cells to increase VitBAg capture from both endosomal and extracellular sources, enhancing delivery of the ligand to the ER for MR1 binding and presentation.

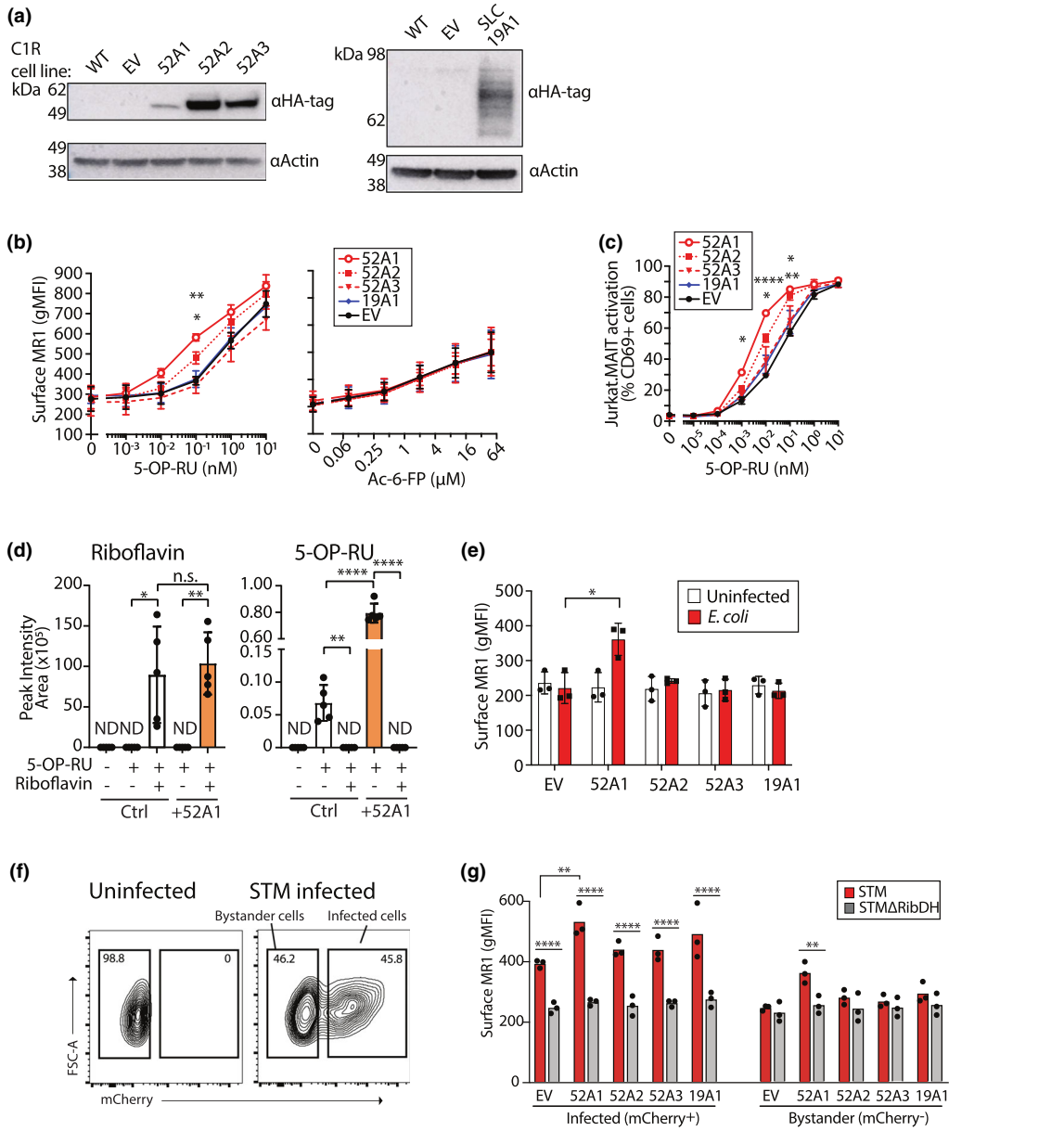

Figure 5. Overexpression of riboflavin transporters enhances MR1-VitBAg presentation. **(a)** C1R5 cells were stably transduced with haemagglutinin (HA) tag-fused transporters: those for riboflavin (SLC52A1, −52A2, −52A3) or folate (SLC19A1), compared to wildtype (WT) cells or those transduced with just the empty vector (EV). Each was subjected to immunoblotting for the HA or actin. **(b)** Cell lines from **(a)** were subjected to a pulse assay with the indicated amounts of 5-OP-RU (left) or Ac-6-FP (right), then cell-surface MR1 was detected by flow cytometry. **(c)** Cell lines from **(a)** were subjected to a pulse assay with the indicated amounts of 5-OP-RU, then cells were co-incubated with Jurkat.MAIT cells for 16 h. Jurkat.MAIT cell activation was evaluated measuring CD69 by flow cytometry. **(d)** Control C1R cells (Ctrl) or those stably transduced with the riboflavin transporter SLC52A1 (+52A1) were first incubated with or without riboflavin (1 mM) for 30 min then with or without 5-OP-RU (10 μM) for 1 h, then cell lysates were subjected to HILIC-MS and 5-OP-RU and riboflavin uptake were measured. **(e)** Overexpressing cell lines were infected with *Escherichia coli* at 1 multiplicity of infection (MOI) for 16 h compared to uninfected, then cell-surface MR1 was detected by flow cytometry. **(f)** Representative flow cytometry plot of C1R cells uninfected (left) or infected with mCherry-expressing *Salmonella enterica* serovar Typhimurium (STM) at MOI 25 (right). Within the infected condition, cells harboring intracellular STM are differentiated from those without (bystander cells) with mCherry fluorescence. **(g)** Cell lines from **(a)** were infected with wildtype STM (MOI 25) or those deficient for riboflavin synthesis (STMΔ*ribDH*; MOI 50) for 16 h, then cell-surface MR1 was detected by flow cytometry. Data shown is the mean ± SD of three independent experiments **(b, c, e, g)**, 5 replicates **(d)**, or the representative images of three independent experiments **(f)**. Statistical significance was calculated using two-way ANOVA using Šídák's multiple comparison test comparing each group against each other where $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$.

DISCUSSION

MAIT cells have been implicated in immunity against viruses,[33] bacteria,[5] protozoans,[34] and cancer.[35] They also play roles in tissue repair,[36] allergy,[37] and autoimmunity.[38] Characterization of the mechanisms that prime and regulate MAIT cell activity in each of these scenarios may aid the design and development of new therapeutic agents. Ideally, such therapeutics should be specific, for example promoting anticancer MAIT cell activation without causing autoimmunity.[39] This is why it is important to identify the main drivers of MAIT reactivity in each disease, as it may allow design of drugs that target specific MAIT functions. In some cases, MAIT activation is mediated only by cytokines,[40] but in others, it requires MAIT TCR recognition of MR1-presented ligands. Among the latter, the ligands can be microbial products, or self-metabolites produced by, for example cancer cells.[5,41] Here, we provide insights into the mechanisms specifically involved in presentation of the most studied and arguably most functionally relevant family of MR1 ligands: VitBAgs.

The first conclusion of our study is that VitBAg presentation is facilitated by trafficking through the cytosol of antigen presenting cells. Thus, cells infected with cytosolic bacteria presented VitBAg more efficiently than cells where the bacteria were constrained to the lumen of endosomes, even when the total amount of bacteria in the two cell groups was equivalent. MR1 can traffic from the plasma membrane to endosomes, bind VitBAg within those compartments and recycle back to the cell surface.[15] However, the physiological relevance of this pathway of MR1 presentation has been put into question because the proportion of endocytosed MR1 molecules that undergo recycling has been estimated to be only ~5%,[11,19] and mutations in the MR1 cytosolic tail that promote recycling diminish, rather than promote, VitBAg presentation.[11] The alternative location where MR1 could bind VitBAgs is the ER, and several studies have shown this is where most MR1-VitBAg complexes form.[11,14,22,23] Mechanisms that promote the maintenance of a ligand-receptive pool of empty MR1 molecules within the ER support this notion,[14,15] but this implies that VitBAg must enter the ER lumen, which would seem to involve one of two possible routes. First, a retrograde transport pathway from endosomes to the Golgi complex and then to the ER, which would not require VitBAg translocation across membranes.[42] Second, transfer through the ER limiting membrane, which would require the VitBAg to first be in the cytosol. Our results favor the second scenario. The second line of evidence that argues against a prominent role for endosomes in MR1 binding of bacteria-derived VitBAg is the observation that overexpression of a riboflavin transporter (SLC52A1) enhanced their presentation, even when VitBAg was produced within endosomes harboring bacteria. Such a result is more difficult to explain if MR1-VitBAg complex formation occurred within endosomes than if it involved transport to the cytosol across the endosomal membrane. Strikingly, SLC52A1 overexpression also enhanced presentation of VitBAg by noninfected neighboring cells. The most likely explanation for this result is that the cells harboring bacteria released VitBAg to the extracellular medium, which was then captured and presented by noninfected cells. Extracellular VitBAg presentation was not impaired by endocytosis inhibitors that reduced dextran uptake, suggesting they were more likely translocated from the extracellular medium into the cytosol by a plasma membrane transporter. Indeed, these results suggest that even cells harboring bacteria in their endosomes may transport VitBAg to their cytosol not from the endosomal lumen but from the extracellular environment, although this remains speculative. In any case, a corollary of these results is that VitBAg, either produced within endosomal compartments or contained in the extracellular environment, enter the cytosol using a specialized transport mechanism, subsequently translocate to the ER lumen, and then bind to the ER-resident pool of MR1 molecules. Manipulations that increase or reduce the amount of VitBAg that accesses the cytosol of MR1 presenting cells, regardless of the effect they may have on VitBAg production in endosomes, or on delivery of VitBAg to these compartments, would increase or impair MAIT cell responses, respectively.

Analysis of the effect of riboflavin, FMN, and FAD on VitBAg presentation provided additional insights into the mechanisms of VitBAg uptake. These compounds competed with MR1 presentation of 5-OP-RU but not 6-FP, implying their inhibition was ligand-specific. Their effect might be to compete for MR1 binding in the ER or to inhibit uptake. A recent study found that FMN and FAD can be converted to riboflavin by the cell-surface ectoenzyme CD73 and alkaline phosphatase.[43] CD73 has a broad expression, and B cells express CD73[44] and alkaline phosphatase,[45] and since C1R cells are a B-cell derived cell line, it is likely they also express these molecules. Therefore, inhibition of VitBAg uptake by FMN and FAD might be caused by riboflavin rather than by the molecules themselves.

Quantitation of 5-OP-RU content in cells incubated with this VitBAg in the absence or presence of riboflavin, using HILIC, confirmed riboflavin inhibited 5-OP-RU uptake. This inhibition would be difficult to explain if the mechanism of 5-OP-RU capture were fluid-phase endocytosis. The alternative and more likely explanation is that riboflavin interfered with a mechanism that

transports 5-OP-RU across the plasma membrane, from the extracellular medium to the cytosol. This result adds support to the notion that MR1-VitBAg complex formation does not occur in endosomes, and more importantly, demonstrates that VitBAg presentation depends on an active transport mechanism with limited capacity. This mechanism represents a potential target for synthetic competitive inhibitors of the transporter.

Overexpression of two of the three known transporters of riboflavin, SLC52A2, and especially SCL52A1, enhanced 5-OP-RU capture and MR1 presentation. A conclusion of this study is therefore that a (hypothetical) allosteric enhancer of these transporters would promote VitBAg presentation and MAIT cell immunity. However, whereas elimination of the three riboflavin transporters compromised cell viability in media with low concentrations of this essential vitamin, it did not prevent 5-OP-RU presentation. This suggests there are redundant mechanisms for 5-OP-RU transport that still work even in cells that lack the canonical riboflavin transporters. This is a plausible explanation because cells lacking all three riboflavin transporters survived in medium containing the standard concentration of riboflavin found in synthetic media, indicating they could capture the vitamin with some alternative, less-efficient transporter. Identification of such a transporter remains an important objective for future studies, but we speculate that the redundancy of VitBAg capture will make it difficult to design strategies to inhibit VitBAg presentation.

In summary, our results help to narrow down the range of potential mechanisms and molecules that might be targeted with therapeutics. First, transporters of VitBAg from the extracellular environment to the cytosol may be targets. Second, mechanisms that affect the amount of VitBAg that occurs in the cytosol of MR1 presenting cells, which may include proteins that increase their stability, could be possible targets too. A third potential target is a yet-unidentified transporter of VitBAg from the cytosol to the lumen of the ER.

MATERIALS AND METHODS

Study design

The objective of this study was to describe new routes in the internalization pathways for VitBAg antigens used for MR1 presentation, previously thought to be related to endosomal pathways. Our work unveils the activity of SLC52A riboflavin transporter family members and extends the scope to a cytosolic route for VitBAg antigen presentation. We used several cellular biology and immunology, molecular biology, and mass spectrometry approaches to study MR1 VitBAg antigen transport and presentation. All experiments were repeated at least three times, using technical duplicates on each repeat for cell line experiments.

Cell cultures and media conditions

All DMEM media and variations were produced in-house at the Media Preparation Unit (MPU) at the Doherty Institute. The C1R, THP-1, and HeLa cell lines were grown and passaged using DMEM, supplemented with FCS (Gibco), L-glutamine (Gibco), and penicillin/streptomycin (MPU). For soluble VitBAg antigen related experiments and riboflavin starvation experiment, we used a modified DMEM that was riboflavin free (rf) and folate free (ff) (rf-ff DMEM) or just folate-free (ff DMEM). These were supplemented with FCS previously dialyzed (dFCS) using a Slide-A-Lyzer G2 cassette (Thermo Fisher) following product instructions, L-glutamine (Gibco), and penicillin/streptomycin. When required, riboflavin (Sigma) was used to restore the required concentrations (1 μM or 25 nM) in rf-ff DMEM or rf DMEM accordingly.

Mutant cell lines generation

C1R cells expressing SLC52A-HA family or SLC19A-HA were transduced with the pLenti-puro vector, in which the coding sequence the respective SLC transporter sequence fused to a Hemagglutinin tag (HA) was inserted. Cells were then selected by using puromycin at 1 μg/mL. Expression of transporters fused to HA tag was verified by western blot. To generate C1R knockout cells for SLC52A transporters, we used C1R cells expressing Cas9 endonuclease (C1R.Cas9) to edit the genes of the respective transporters using sgRNAs inserted into FgH1tUTG vector (Addgene), with GFP as a transduction reporter. Expression of these sgRNA was induced by tetracycline to produce the gene deletions. GFP expression was used to sort bulk populations of effectively transduced cells. Single cell clones were obtained by sorting bulk populations and sequencing the clones to identify those containing only frameshift mutations in the respective transporter genes. Selected clones were then used to generate the final cell lines. The following guide RNA sequences directed to exon 3 of each gene were used to edit cells: sgCtrl_1 (ACGGAGGC TAAGCGTCGCAA), SgCtrl_2 (CGCTTCCGCGGCCCGTTC AA), SLC52A1_sg2 (TCAGGGCCTGAACTTCAACT, sense), SLC52A1_sg3 (AAGAAAGACCGTAAGAAAGG, antisense), SLC52A2_sg1 (AATGACCGTAAGAAGCGAGG, antisense), SLC52A2_sg2 (ACGCTCAAGGAAGTCGAGCG, antisense), SLC52A3_sg1 (CCCAGGAGGTCATATTCCAG, antisense), SLC52A3_sg2 (AGATGATGGGCACTTCGGAA, antisense). The following primers were used to amplify the genetic region targeted by different guide RNA: SLC52A1 (F: CCTAACTC TGGCCTTGGTGT, R: CTGCAATGGCAAAGCCTCTT, 421 pb amplicon), SLC52A2 (F: TACGTCTCTGTGCTTGTGGCTC, R: CTCTTGCAGTGGTGAGGACTCTT, 609 pb amplicon), SLC 52A3 (F: CTGGTCTGCGTCTTCGGAAT, R: TCAGTGAC ATTGACGCAGGT, 491 pb amplicon).

Western blot

Samples were lysed with lysis buffer containing 0.5% IGEPAL CA-630 (Sigma-Aldrich), 50 mM Tris–HCl (pH 7.4), 5 mM MgCl2, Complete Protease Inhibitor Cocktail (Roche). Nuclei were separated by centrifugation at 13000 × g for 10 min. Lysates were incubated on ice for 30 min. Bolt LDS sample buffer (Life Technologies) and dithiothreitol were added to cell lysates and incubated for 10 min at 98°C. Samples were run in a Bolt SDS acrylamide/bis-acrylamide gel electrophoresis (Biorad) following manufacturer's instructions. Proteins were transferred to a nitrocellulose membrane using an iBlot system (Invitrogen), membrane was blocked using ECL blocking prime reagent (GE Healthcare) for 1 h at RT. Anti-HA tag antibody clone C29F4 (CellSignaling) was incubated for 18 h in blocking buffer at 4°C. Then membrane was washed thrice with PBS-Tween 0.1% and incubated with Donkey anti-Rabbit-HRP (GE Healthcare) diluted in blocking buffer for 1 h at RT. Membrane was washed thrice with PBS-Tween 0.1% and signal was developed with ECL Select Western Blotting Detection Reagent kit (GE Healthcare) in an Imager Amersham 600 (GE Healthcare).

MR1 ligands, vitamins, and the pulse assay

5-OP-RU was synthesized,[21,46] stored, and quantified[47] as DMSO solutions as previously described, with care taken to minimize its exposure to water. Uptake of 5-OP-RU was performed by incubating the cells with the indicated concentrations of VitBAg antigen diluted in rf DMEM, supplemented with dFCS, L-glutamine, and penicillin/streptomycin, for 1 h at 37°C. All other MR1 ligands or vitamins were stored in 0.1 M NaOH, which were diluted in cell culture media at least 1000-fold and thus the final amount of NaOH for cell culture was 100 nM or less. The same amount of NaOH (or DMSO in the case of 5-OP-RU) was used for vehicle controls.

Pulse assays with 5-OP-RU at 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 μM, or Ac-6-FP at 0, 0.016, 0.08, 0.4, 2, 10, 50 μM were performed by incubating cells with the respective VitBAg diluted in rf and/or ff DMEM, supplemented with dFCS, L-glutamine and penicillin/streptomycin, for 30 min at 37°C or 4°C. Then, VitBAg antigen excess was removed by washing twice with PBS. Cells were incubated with VitBAg antigen free media for 2.5 h and surface MR1 expression was analyzed by flow cytometry. Inhibition assays were performed by incubating cells for 30 min at 37°C in cell media in the presence of inhibitor. A final concentration of 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 μM 5-OP-RU or 0, 0.016, 0.08, 0.4, 2, 10, 50 μM Ac-6-FP was then added for 30 min at 37°C, in the presence of inhibitor, excess VitBAg antigen was removed by washing (x2) with PBS. Cells were incubated in VitBAg antigen free media for 2.5 h at 37°C, followed by flow cytometry staining. Inhibition of Jurkat.MAIT (clone AF-7) GFP^+ cell activation was performed by incubating C1R cells with inhibitors for 30 min at 37°C, then adding 5-OP-RU to obtain indicated concentrations. Excess of ligand was removed by washing washed twice and VitBAg antigen loaded C1R cells were co-incubated with Jurkat.MAIT cells in VitBAg antigen free supplemented DMEM for 16 h. Riboflavin, flavin mononucleotide, flavin adenine mononucleotide, thiamine, D-pentose, adenosine, guanosine, cytidine, thymidine, uridine, inosine, adenine, guanine, cytosine, thymine, uracil, cytochalasin D, and EIPA were obtained from Sigma.

Flow cytometry

Surface MR1 expression was detected by incubating treated cells with an antibody anti-MR1 clone 8F2.F9 directly conjugated to AF647 (WEHI) or conjugated to biotin (WEHI), while biotin was detected using Streptavidin-AF647 (Thermo Fisher). Dead cells were identified by staining with Live/Dead cell marker Live/Dead Fixability Dye eFluor780 (Thermo Fisher). Jurkat.MAIT cell activation was detected by staining cells with dead cell marker, anti-CD69-Pacific Blue clone FN50 (Biolgend), anti-HLA-ABC-Brilliant Violet 605 clone W6/32 (Biolegend) and anti-CD3-PE clone HIT3a (Invitrogen). For counting cells after riboflavin starvation experiments, cells were incubated with Sphero Blank Calibration Particles 6.0–6.4 μm (BD biosciences). All FACS data was obtained on a Fortessa II flow cytometer using the FACS DIVA software. Washings after staining incubations were performed twice with EDTA/BSS 2% FBS buffer. All FACS analyses were performed using the FlowJo 10.7.1 software. For all experiments, the naturally low endogenous level of MR1 was measured by flow cytometry. Robust detection of these low levels of MR1 requires the use of a bright fluorophore such as Alexa Fluor 647, ensuring that the intensity of the laser is optimized in the flow cytometer settings.

Bacterial infection models

Salmonella enterica serovar Typhimurium and *Escherichia coli* MOI were calculated based on the reference OD600: $1 = 5 \times 10^8$ bacteria/mL. Required bacterial cells were washed thrice with PBS before using them on cell lines. Infections of C1R.SLC52-HA cells with *Salmonella enterica* serovar Typhimurium strains SL1344 or HW101 in cells were performed by incubating cells with an MOI 25 or 50, respectively in DMEM supplemented with FCS, L-glutamine without antibiotics for 1 h at 37°C. Then, cells were washed twice with cell media supplemented with gentamycin at 40 μg/mL (Sigma), followed by an incubation in this media for 18 h at 37°C. After this, surface MR1 was evaluated by flow cytometry. Infection of C1R.SLC52-HA cells with *E. coli* strain DH5α was performed by infecting these cells with at a MOI 1 in media without antibiotics for 18 h at 37 °C. For HeLa cell infections with *Salmonella enterica* serovar Typhimurium strains SL1344 WT, Δ*sopF* or Δ*sifA* were transformed with the PuhpT-*gfpova* plasmid. HeLa cells were infected using STM SL1344 WT, Δ*sopF* or Δ*sifA* an MOI 50 of the corresponding strains for 1 h at 37°C, cells were washed twice with cell media supplemented with gentamycin at 40 μg/mL to then incubate with media, supplemented with

dFCS and L-glutamine for 7 h. After this, surface MR1 expression was evaluated by flow cytometry.

PCR and RT-qPCR

Populations identified during STM infection on HeLa cells were sorted in a MoFlo Astrios cell sorter. For each population, cells were pelleted, and their RNA extracted using RNAeasy Kit (Qiagen), cDNA was generated using SensiFAST cDNA synthesis kit (Bioline) following product instructions in a MasterCycler Nexus thermocycler (Thermo). Genomic DNA for sequencing was extracted using QuickExtract DNA extraction Solution (Lucigen). MR1 gene expression was evaluated using TaqMan PreAmp Master Mix (Thermo Fisher Scientific) and TaqMan Fast Advanced Master mix (Thermo Fisher). Probes used were MR1 (Hs01042278_m1) and RPL27 (Hs03044961_g1). Gene expression was normalized to the housekeeping gene RPL27 and presented as $2 - \Delta\Delta Ct$ arbitrary units of mRNA expression levels. RT-qPCR was performed in a Quant Studio 7 machine.

Mass spectrometry

Soluble 5-OP-RU samples were prepared by diluting it in DMSO and used to generate a standard curve up to 10 μM. For every sample 1×10^7 C1R.sgCtrl-1, C1R.ΔSLC52A2_sg1 and C1R.ΔΔSLC52A1_sg2_A3_sg1 cells per sample were used, these cells were incubated with 10 μM of soluble 5-OP-RU or the corresponding volume of DMSO diluted in rf-ff DMEM supplemented with FBS, L-Glutamine and penicillin/streptomycin. Five replicates of each condition were submitted to Metabolomics Australia (The University of Melbourne) for metabolomics analysis. The cell pellets were solubilized in 120 μL of ice-cold 2:2:1 Acetonitrile:Methanol: Milli-Q Water containing 2 μM ^{13}C-sorbitol, ^{13}C,^{15}N-valine, ^{13}C,^{15}N-AMP, ^{13}C,^{15}N-UMP as internal standards. The cellular pellet was vigorously vortexed and incubated for 10 min at 4°C on a Thermomixer at 1000 RPM to ensure complete lysis of all cell membranes. The cellular debris was removed by centrifugation at 4°C for 10 min and the supernatant collected for high-performance liquid chromatography mass spectrometer (LCMS) analysis. Additionally, a four-point standard curve consisting of 1.0, 0.1, 0.01, and 0.001 μM was used for the quantitation of 5-OP-RU was generated by serial dilution into the extraction solvent.

Polar metabolite separation was performed on an Agilent Technologies 1200 series HPLC system (Agilent Technologies, Santa Clara, US, USA) on a SeQuant ZIC–pHILIC (5 μm polymer) PEEK 150 × 4.6 mm metal-free HPLC column maintained at 25°C with solvent A: 20 mM ammonium carbonate (pH 9.0; Sigma–Aldrich) and solvent B: 100% Acetonitrile, with an injection volume of 10 μL and a flow rate of 300 μL/min. Samples were stored in an autosampler maintained at 4°C. The gradient run was as follows: time (t) = 0.0 min, 80% B; t = 0.5 min, 80% B; t = 15.5 min, 50% B; t = 17.5 min, 30% B; t = 18.5 min, 5%; t = 21.0 min, 5% B; t = 23–33 min, 80% B.

Mass spectrometry analysis was performed on an Agilent Technologies 6545 series quadrupole time of flight mass spectrometer (QTOF MS). The LC flow was directed to an electrospray ionization source (ESI) where metabolite ionization was performed with a capillary voltage of 2500 V, a drying gas (N2) pressure of 20 psig at 150°C with a gas flow rate of 10 L/min and a sheath gas temperature of 300°C. Fragmentor and skimmer cap voltages were set at 125 V and 45 V respectively.

Samples were analyzed in the same analytical batch and randomized with a QC every 5 samples. Data processing and peak curation was performed using Agilent MassHunter Quantitative Analysis software (Version B.07.00/Build 7.0.457.0). Level 1 Metabolite identification according to the Metabolite Standard Initiative[48] was based on matching accurate mass, retention time and MS/MS spectra to the 550 authentic standards in the MA in-house library. Statistical analysis was performed using median-normalized and log-transformed data on the web-based platform MetaboAnalyst.[49]

Statistical analysis

Statistical analysis was performed using One-Way or Two-Way analysis of variance (ANOVA) with multiple comparisons and Tukey (One-way) or Šídák's (Two-Way). Statistical analysis and graphs were performed on the Graphpad Prism 9 software. P values were represented as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. Results represent mean ± SD or SEM, indicated on each Figure.

SIGNIFICANCE

Which cellular route do Vitamin B-related Antigens (VitBAg) follow to reach ligand-receptive histocompatibility complex class I-related protein 1 (MR1), and which proteins facilitate antigen presenting cells to capture VitBAg? In the MR1-MAIT cells field, these fundamental answers remain incomplete. Here, we show that VitBAg follows a cytosolic route to reach ligand-receptive MR1. We discover that riboflavin is a specific inhibitor of 5-OP-RU capture and presentation by MR1. Our results show that riboflavin transporters, while not essential for VitBAg capture, can transport 5-OP-RU and bacterial-derived VitBAg specifically, promoting their MR1 presentation to MAIT cells. Our work shows evidence of a cytosolic route and identifies proteins that can directly promote VitBAg capture and MR1 presentation leading to MAIT cells' activation.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.