Ligand-dependent downregulation of MR1 cell surface expression

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The antigen-presenting molecule MR1 presents riboflavin-based metabolites to Mucosal-Associated Invariant T (MAIT) cells. While MR1 egress to the cell surface is ligand-dependent, the ability of small-molecule ligands to impact on MR1 cellular trafficking remains unknown. Airing from an in silico screen of the MR1 ligand-binding pocket, we identify one ligand, 3-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido)propionic acid, DB28, as well as an analog, methyl 3-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido)propionate, NV18.1, that down-regulate MR1 from the cell surface and retain MR1 molecules in the endoplasmic reticulum (ER) in an immature form. DB28 and NV18.1 compete with the known MR1 ligands, 5-OP-RU and A6-66P, for MR1 binding and inhibit MR1-dependent MAIT cell activation. Crystal structures of the MAIT T cell receptor (TCR) complexed with MR1-DB28 and MR1-NV18.1, show that these two ligands reside within the A'-pocket of MR1. Neither ligand forms a Schiff base with MR1 molecules; both are nevertheless sequestered by a network of hydrophobic and polar contacts. Accordingly, we define a class of compounds that inhibits MR1 cellular trafficking.

Macosal-Associated Invariant T (MAIT) cells are a subset of evolutionarily conserved nonmajor histocompatibility complex (MHC)-restricted T cells, which are very abundant in human mucosal tissues, in peripheral blood, and in the liver (1, 2). Similar to type I NKT cells, human MAIT cells express a semi-invariant T cell receptor (TCR) composed of the Vα7.2 chain rearranged mainly to Jβ chains, mostly TRBV6, TRBV13, and TRBV20 (3, 4). MAIT cells recognize small microbial metabolites presented by the monomorphic MHC class I-related molecule, MR1 (1, 2). The physiological roles of MAIT cells remain unclear, but they are known to be involved in protective immunity (2, 5–7), possibly through modulation of innate and adaptive immune responses (8, 9). Moreover, the role of MAIT cells in cancer (10) and inflammatory diseases, such as obesity (11), diabetes (12), multiple sclerosis (13), and inflammatory bowel disease (14), has been highlighted, and recent reports have suggested they may also play a role in tissue repair (15, 16). Activation of MAIT cells induces the production of various proinflammatory cytokines, predominantly IFN-γ, TNF-α, IL-2, and IL-17 (17, 18), and their potent cytolytic activity allows them to kill infected cells (19). Unlike MHC molecules, MR1 does not constitutively present antigens, but is found in the endoplasmic reticulum (ER) of all cells in a ligand-receptive conformation (20). The potency of known MAIT cell agonists appears to correlate with their ability to form a Schiff base with MR1 Lys43 located within the A'-pocket, thus allowing MR1 to egress to the cell surface, where the presence of a ribityl moiety in the covalently bound agonist allows for an interaction with the MAIT TCR (21–23). To date, the strongest MAIT cell agonists are 5-(2-oxopropylidenamino)-6-ribitylamino-nicuracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-ribitylamino-nicuracil (5-OE-RU), both pyrimidine-based intermediates along the riboflavin biosynthetic pathway (24). Several bacterial and fungal species synthesize riboflavin (23), and MAIT cells have been shown to possess MR1-dependent antimicrobial activity against infected antigen-presenting cells (5, 6). Conversely, vitamin B9 metabolites (including the folate acid derivative folinamic acid, 6-FFP and its acetylated derivative Ac6-66P (25, 26)) are strong MR1 binders and induce MR1 expression at the cell surface; however, the resulting complexes do not activate MAIT cells because they lack the ribityl moiety (22). Drug and drug-like molecules (including diclofenac and salicylates) also bind MR1 and either weakly activate or inhibit MR1 egress to the cell surface, thus allowing MR1 to egress to the cell surface, describing nonmicrobial ligands, DB28 and its ester analog NV18.1, which retain MR1 in the ER in an immature ligand-receptive form and competitively inhibit stimulatory ligands. We provide the molecular and functional basis underpinning the interactions of this class of ligands with MR1.

**Significance**

MR1 is a monomorphic major histocompatibility complex (MHC) class I-like molecule that presents ligands to Mucosal Associated Invariant T cells. MR1 antigen presentation at the cell surface is tightly regulated by ligand availability. Although previously described MR1 ligands facilitate translocation of ER-resident MR1 to the cell surface, we describe nonmicrobial ligands, DB28 and its ester analogue NV18.1, which retain MR1 in the ER in an immature ligand-receptive form and competitively inhibit stimulatory ligands. We provide the molecular and functional basis underpinning the interactions of this class of ligands with MR1.

Author contributions: M.S. and V.C. designed research; M.S., W.A., N.V., C.G.-L., C.K., A.W.J.M., J.V.H., L.R.C., and J.R. performed research; D.M.L. contributed new reagents/analytic tools; M.S., D.W., and V.C. analyzed data; and M.S., W.A., N.V., J.R., G.S.B., and V.C. wrote the paper.

The authors declare no competing interest.

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Data deposition: The coordinates of the ternary complexes of MAIT A-F7 TCR-MR1-DB28 and TCR-MR1-NV18.1 have been deposited in the Protein Data Bank (PDB), under accession codes 6PVC and 6PD6.

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MAIT cells (26). However, it remains unknown whether there are other ligands that impact MR1-dependent antigen presentation.

Through an in silico screen, we have identified additional MR1-binding ligands. We describe a ligand that down-regulates MR1 cell-surface expression and provide a molecular basis for its interactions with MR1.

Results

Identification of Nonmicrobial MAIT Cell Agonists. To identify MR1 binding ligands, we performed in silico screening using the crystal structures of the MAIT TCR in complex with MR1-antigen complexes [PDB codes 4L4V and 4LCC (22, 27)]. A total of 44,022 compounds were selected for docking runs, based on searches for fragment size substructures s1-s20 (SI Appendix, Fig. S1 and Supplementary Methods). Compound selection and constraints imposed during docking are detailed in the supplementary methods. Using this strategy, 80 commercial compounds were selected as potential MR1 ligands, of which 52 compounds were pulsed on MR1 overexpressing cells, alongside the canonical MAIT cell ligand 5-OP-RU, synthesized and validated in house (SI Appendix, Fig. S2 A and B). MAIT cell stimulatory activity was observed when THP1-MR1 cells (Fig. 1A and SI Appendix, Fig. S4A) were pulsed with compounds DB5, DB7, DB8, DB12, DB15, DB19, and DB23, whose chemical structures are shown in SI Appendix, Fig. S3. Overall, these compounds were three to nine times less potent than 5-OP-RU (SI Appendix, Fig. S4A). Unlike Ac-6-FP and 5-OP-RU (26), none of the tested compounds induced detectable up-regulation of cell-surface MR1, neither after 5 nor 22 h (Fig. 1B). Presentation was MR1-dependent, as determined using the blocking anti-MR1 26.5 monoclonal antibody (28) (Fig. 1C) and pulsing the compounds on MR1-KO THP1 cells (SI Appendix, Fig. S4B). Consistent with their weaker potency, presentation by THP1 cells required a higher level of MR1 expression (THP1-MR1 WT, required a higher level of MR1 expression (THP1-MR1 WT,)

![Fig. 1. MAIT cell stimulation by the DB series of agonists. (A) THP1-MR1 cells were pulsed with the indicated compounds (100 μM MG, 20 μg/mL DB compounds, 50 ng/mL 5-OP-RU) and incubated with MAIT cells. IFN-γ was measured in the supernatant after 36 h of coculture. Box and whiskers bars, minimum to maximum, with all points indicated; n = 5. (B) The DB series of MAIT cell agonists do not induce MR1 up-regulation. THP1-MR1 cells were pulsed 5 or 22 h with 20 μg/mL of the indicated compounds, 100 μM MG, 1 μg/mL Ac-6-FP, or 1 μg/mL 5-OP-RU. Depicted is the cell surface expression of MR1 at 5 h (black bars) or 22 h (white bars) measured by FACS. Data are mean ± SD of technical duplicates. One experiment is representative of two. (C) MR1-dependent presentation of the DB series of agonists. THP1-MR1 cells were pulsed with the indicated compounds (100 μM MG, 20 μg/mL for DB compounds, 50 ng/mL for 5-OP-RU) and incubated with MAIT cells in the presence of isotype control or blocking anti-MR1 antibodies. IFN-γ was measured in the supernatant after 36 h of coculture. Data are mean ± SD of technical duplicates. One experiment is representative of two. (D) DC were pulsed with the indicated compounds (100 μM MG, 20 μg/mL DB compounds, 50 ng/mL 5-OP-RU) and incubated with MAIT cells. IFN-γ was measured in the supernatant after 36 h of coculture. Average from technical duplicates from three different donors. One experiment is representative of two. (E) Ex vivo MAIT cell activation by the DB series of agonists. Whole blood was stimulated overnight with the indicated compounds. MAIT cell activation, depicted as percentage of CD137 expression, was assessed by flow cytometry. Box and whiskers bars, minimum to maximum, with all points indicated; n = 7. (F) TCR chain expression influences reactivity to DB MAIT cell agonists. MAIT cells expressing TRBV20.1, TRBV13S2, and neither of those two chains (DN) were sorted from a single donor and incubated with THP1-MR1 pulsed with the indicated compounds (100 μM MG, 20 μg/mL for DB compounds, 50 ng/mL for 5-OP-RU). UP, unpulsed. IFN-γ was measured in the supernatant after 36 h of coculture. Data are mean ± SD of technical duplicates. One experiment is representative of two.
whereas THP1 cells nonoverexpressing MR1 were unable to present any of the DB compounds (SI Appendix, Fig. S4B). In addition, presentation was reduced or abrogated when THP1 cells expressing GPI-linked molecules were used (SI Appendix, Fig. S4B), suggesting internalization and possibly endo-lysosomal loading is required. However, we were unable to detect any MAIT cell activation by fixed THP1-MR1, even after pulsing with the potent agonist 5-OP-RU; therefore, we did not investigate intracellular trafficking further. Compounds DB5, DB12, and DB19 were also presented by monocyte-derived dendritic cells (Fig. 1D). In this experimental setting, MAIT cell activation was also MR1-dependent (SI Appendix, Fig. S4C). We next tested the compounds on unfractionated cells in whole blood and identified MAIT cells by Vα7.2 and CD161 co-staining. MAIT cell activation (measured by CD137 up-regulation) with compounds DB7, DB8, DB12, and DB19 was observed in some, but not all, of the seven donors tested (Fig. 1E); this may reflect pairing of different TCR β-chains with the canonical MAIT TCR α-chain (3). Indeed, in one donor, we observed a lower response by MAIT cells expressing the TRBV13S2 chain as compared with the TRBV20.1 chain or neither of those two chains (Fig. 1F). We also confirmed TCR-mediated recognition of some of the DB compounds using Jurkat cells transduced with a MAIT TCR composed of the
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DMSO

10^3

10^5

10^4

n.s.

10^3

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DB28 Down-Regulates Cell Surface Expression of MR1. When testing the 52 compounds for ligand-induced MR1 up-regulation, we noticed that, unlike Ac-6-FP and 5-OP-RU, which potently up-regulate MR1 cell-surface expression (24, 25), compound DB28 (3)-(2,6-dioxo-1,2,3,6-tetrahydroprypimidin-4-yl)formamido)propanoic acid, Fig. 24) not only failed to up-regulate MR1 at the cell surface of THP1-MR1 cells but also reduced its expression to almost background levels of staining, using the anti-MR1 monoclonal antibody 26.5 (Fig. 2B). The structural formula of compound DB28, and its ester derivative NV18.1 (methyl 3-

(2,6-dioxo-1,2,3,6-tetrahydroprypimidin-4-yl)formamido)prop-

anate), are shown in Fig. 24. 5-OP-RU and Ac-6-FP covalently bind within the MR1 A'-pocket by forming a Schiff base with MR1 Lys43 (24, 25), which triggers MR1 egress from the ER and trafficking to the cell surface (20). DB28 has a terminal carboxylic acid, while the NV18.1 is its methyl ester analog; thus, without undergoing reduction, neither is able to form a Schiff base with MR1. Cell-surface MR1 down-regulation by DB28 and NV18.1 was observed with two different conformation-specific MR1 antibodies, 26.5 and 8F2.F9 (30, 31) (Fig. 2C), where NV18.1 was less potent than DB28 (Fig. 2D). MR1 down-regulation was observed when the compounds were tested in both myeloid cells (THP1-MR1) and EBV-transformed B cells (C1R-MR1) (Fig. 2D). The effect was specific for MR1, as no down-regulation of MHC-I or CD1d molecules was observed at the surface of THP1 cells (SI Appendix, Fig. S5).

The MR1 transcript is ubiquitous, but expression on primary cells is low (32, 33). Nevertheless, we observed down-regulation of basal and 5-OP-RU-induced surface expression of MR1 by DB28 in primary B cells and monocytes freshly isolated from four healthy donors (Fig. 3), confirming results previously obtained with THP1 cells overexpressing MR1.

We next tested whether DB28 inhibits the up-regulation of MR1 induced by other ligands. As shown in Fig. 4 A and B, DB28 abrogated Ac-6-FP- or 5-OP-RU-induced up-regulation of MR1 surface expression, and this effect was stronger when DB28 was in molar excess, suggesting competition for MR1 binding.

canonical TCR α-chain paired with TRBV20.1 or 6.4 (SI Appendix, Fig. S4 D and E) (29). In conclusion, we have defined a series of compounds that bind MR1 and can activate, through MR1-TCR interaction, MAIT cells expressing a variety of TCR β chains.

Fig. 3. DB28 down-regulates MR1 from the cell surface of primary cells. CD2-depleted PBMC were incubated overnight with MG (50 μg/mL), 5-OP-RU (1 μg/mL) with or without DB28 (20 μg/mL), and MR1 expression on the surface of gated live B cells (A and B) or monocytes (C and D) was determined by flow cytometry. Cumulative data of geometrical mean fluorescence intensity of four different blood donors (A and C), representative FACs histograms (B and D). The background staining of isotype control is shown in black.
We added DB28 either concurrently with, 2 h before, or 2 h after 5-OP-RU. In all cases, DB28 reduced MR1 cell surface expression. This effect was reversible, since it persisted as long as DB28 was kept in culture for the duration of the assay and not washed away after the first 5 h (Fig. 4C). The reversibility of the DB28 effect prompted us to investigate the contribution of protein synthesis, which is not required for ligand-induced up-regulation of MR1 surface expression (20). At steady state, DB28 down-regulated surface expression of 90% of MR1 molecules, whereas only 60% of MR1 molecules were down-regulated in the presence of cycloheximide. Likewise, in the presence of 5-OP-RU, DB28 down-regulated 70% of MR1 molecules, but only 50% when cells were also treated with cycloheximide (although this difference did not reach statistical significance, Fig. 4D). To avoid potential off-target effects of cycloheximide, we tested the epithelial cell line BEAS2B expressing a tetracycline-inducible MR1 construct tagged with GFP (34, 35). Upon doxycycline treatment, more than 90% of cells became GFP positive and 10% to 15% of cells expressed cell surface MR1 (SI Appendix, Fig. S6). There was a trend toward lower MR1 down-regulation in the absence of doxycycline; however, it did not reach statistical significance (Fig. 4E). In conclusion, we observed selective down-regulation of MR1 cell surface expression by DB28 and NV18.1, which was only marginally affected by blocking protein synthesis.

Requirements for DB28 Down-Modulation of MR1 Expression. As previously shown (20), the Lys43Ala mutation facilitates the release of MR1 molecules from the ER even in the absence of vitamin B metabolites. To dissect the molecular mechanism by which DB28 induces MR1 down-regulation, we generated THP1 cells expressing Lys43Ala-mutated MR1 molecules (SI Appendix, Fig. S7) (20). For these experiments, we used MR1 KO THP1 cells (36) to avoid residual activity of WT MR1 molecules on the cell surface with Ac-6-FP (20) (Fig. 6C and SI Appendix, Fig. S8A). In contrast, in the presence of DB28, they remained in the ER/Golgi compartments. Furthermore, MR1 molecules immunoprecipitated with 26.5 antibody [which recognizes folded MR1 molecules (28)] remained EndoH sensitive in the presence of DB28, as expected from their ER localization. In the presence of 5-OP-RU, they acquired partial EndoH resistance, which was abrogated by coinubcation with DB28 (Fig. 6D and SI Appendix, Fig. S8B). In conclusion, DB28 retains the immature form of MR1 within the ER.

**DB28 Prevents MAIT Cell Activation by Agonist Ligands.** Upon recognition of their cognate antigen via the TCR, MAIT cells release cytokines in an MR1-dependent manner (6, 19). In agreement with a lack of effect of DB28 on CD1d expression (SI Appendix, Fig. S5), INKT cell activation was unaffected when THP1 cells were pulsed with αGalCer and DB28 (SI Appendix, Fig. S9A). Consistent with the ability of DB28 to down-regulate MR1 cell surface expression even in the presence of MAIT cell agonists (Fig. 4B), we observed dose-dependent inhibition of MAIT cell activation when DB28 was pulsed on THP1-MR1 cells with an agonist ligand (Fig. 7A and B). Likewise, presentation of vitamin B metabolites by epithelial cells infected with bacillus Calmette-Guérin (Fig. 7C and SI Appendix, Fig. S9B) or by peripheral blood mononuclear cells (PBMC) infected with Escherichia coli (Fig. 7D and SI Appendix, Fig. S9C) was significantly reduced in the presence of DB28. This effect was specific to MR1-dependent stimulation, as activation of MAIT cells with phytohemagglutinin was not affected by DB28 (Fig. 7C and SI Appendix, Fig. S9B), nor was activation of bystander CD161hi T cells upon bacterial exposure (SI Appendix, Fig. S9D). We confirmed that even in bacillus Calmette-Guérin-infected BEAS2B epithelial cells (SI Appendix, Fig. S10A and B) and E. coli-infected myeloid cells (THP1; SI Appendix, Fig. S10 C and D), DB28 down-regulated cell surface MR1 (SI Appendix, Fig. S10A and C), but not total MR1 (SI Appendix, Fig. S10 B and D).

MR1 molecules are highly evolutionarily conserved, with 90% sequence homology between the a1 and a2 domains of humans and mice (32). In vitro experiments with murine bone marrow-derived DC confirmed the ability of DB28 to inhibit MR1-dependent human MAIT cell activation (SI Appendix, Fig. S11A). We next injected i.v. DB28 to assess its effect on 5-OP-RU-dependent in vivo MAIT cell activation (SI Appendix, Fig. S11B). Despite a 9 to 90 range of molar excess of DB28, we did not observe inhibition of MAIT cell activation, which we hypothesize might be a consequence of the rapid clearance of the compound, as it does not form a Schiff base with MR1 molecules.

**DB28 Retains MR1 in the ER in an Immature Form.** To investigate the fate of MR1 on incubation of cells with the ligand DB28, we fixed and permeabilized the cells to determine total MR1 content by flow cytometry. Staining with a polyclonal anti-MR1 antibody revealed the total MR1 content was unaffected, thus ruling out degradation of MR1 molecules (Fig. 6A); this was further confirmed with the epithelial cell line, BEAS2B, expressing a tetracycline-inducible MR1 construct tagged with GFP (34, 35) or constitutively expressing GFP-tagged MR1 molecules. In both cell lines, in the presence of DB28, we observed down-regulation of MR1 from the cell surface (Fig. 4E and SI Appendix, Fig. S6B, respectively), but the total GFP content remained unaffected (Fig. 6D and SI Appendix, Fig. S6C, respectively). Consistent with these findings, when we sampled the intracellular distribution of MR1 molecules by confocal microscopy, in the presence of vehicle, MR1 molecules were preferentially colocalized within the ER and Golgi compartments, as previously reported, while they translocated to the cell surface with Ac-6-FP (20) (Fig. 6C and SI Appendix, Fig. S8A).

**Fig. 5.** DB28 down-regulates GPI-linked MR1 molecules, but not K43A mutants. THP1 MR1 KO cells overexpressing WT MR1 molecules, K43A mutants (A), or GPI-linked MR1 (B) were pulsed for 5 h with the indicated ligands (MG 50 μM, Ac-6-FP 1 μg/mL, 5-OP-RU 5 μg/mL, DB28 20 μg/mL) before staining with anti-MR1 (26.5) antibody. Geo MFI ± SD of technical duplicates are plotted in each graph. Data representative of three experimental replicates.

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In conclusion, these results demonstrate that, in vitro, DB28 acts as a competitive inhibitor for MAIT cell-activating ligands.

**MR1-DB28 and MR1-NV18.1 Display Very Weak Binding to MAIT TCRs.**

We next measured the binding affinity of MR1 loaded with 5-OP-RU, Ac-6-FP, DB28, and NV18.1 ligands toward two MAIT TCRs (A-F7 [TRAV1-2-TRBV6-1] and #6 [TRAV1-2-TRBV6-4] TCRs) (25), using surface plasmon resonance (SPR; Fig. 8). As previously reported (25), the 5-OP-RU agonist exhibited affinities to MAIT TCRs ranging from ∼3 to 10 μM, whereas the folate antagonist Ac-6-FP showed weak binding (97.4 ± 30.6 and 235 ± 67.7 μM to A-F7 and TRBV6-4 TCRs, respectively) (24, 25). Consistent with the absence of the ribityl tail and the lack of MAIT cell activation, both DB28 and NV18.1 revealed extremely low binding to AF-7 TCR (KD = 172.0 ± 36.7 and 200.0 ± 64.0 μM, respectively), while we could not measure binding to TRBV6-4 TCR (KD = ND). Collectively, even if the MR1 complexes with inhibitors DB28 and NV18.1 made it to the cell surface, they would exhibit very weak affinities to MAIT TCRs, in agreement with their inability to stimulate MAIT cells.

**Crystal Structures of MR1-DB28 and MR1-NV18.1 Complexes Bound to MAIT TCR.**

To gain insight into the molecular basis underpinning MR1 down-regulation by DB28 and NV18.1, and despite the very low affinity of the interaction, as judged by SPR, we were able to crystallize the MAIT A-F7 TCR-MR1-DB28 and TCR-MR1-NV18.1 complexes, consistent with other structural reports with low-affinity ligands (26, 37). Both ternary structures were determined at 2-Å resolution and exhibited unambiguous electron densities within the MR1-antigen binding cleft and at the TCR-MR1-Ag interfaces (SI Appendix, Table S2 and Fig. 9). The overall topology of the two ternary complexes was very similar, whereby the MAIT TCR docks centrally above the MR1-antigen binding cleft and at the TCR-MR1-Ag interfaces (SI Appendix, Table S2 and Fig. 9). The overall topology of the two ternary complexes was very similar, whereby the MAIT TCR docks centrally above the MR1-antigen binding cleft and at the TCR-MR1-Ag interfaces (SI Appendix, Table S2 and Fig. 9).
As expected from their chemical compositions, neither DB28 nor NV18.1 forms a Schiff base with Lys43 of MR1; however, both ligands are clearly visible in the A-pocket of MR1, as evidenced by unbiased omit maps of the ligands (Fig. 9C and D), indicating strong sequestering of the ligands within the MR1 antigen-binding cleft. Here, the carbonyl group of the uracil ring of both ligands is H-bonded to Lys43, which results in Lys43 being positioned closer to the ring structure when compared with that of 5-OP-RU bound to MR1 (Fig. 9E). In addition, the uracil ring of DB28 and NV18.1 adopts a position reminiscent of the ring structure of 5-OP-RU (Fig. 9F–H), whereby its positioning is largely governed by a network of contacts that include hydrophobic interactions with the MR1 aromatic cradle (Tyr7, Tyr62, Trp69, Ile96, and Trp156), as well as H-bonding to Lys43, which results in Lys43 being positioned closer to the ring structure when compared with that of 5-OP-RU bound to MR1 (Fig. 9E). Interestingly, the orientation of the side-chain appendages of DB28 and NV18.1 within the A-pocket differ from that of 5-OP-RU: both side-chains are constrained by an intramolecular H-bond between the amide NH of the side-chain and the carbonyl oxygen of the carboxylic acid (in DB28) or ester (in NV18.1; Fig. 9F and G). This tilted conformation of the ligands’ side-chains is supported by hydrophobic interactions with Ile96, Trp69, and Trp156 residues, and H-bonding with the Tyr152 of the MR1 α2-helix. Further, the two side-chains lean toward and form H-bonds with Arg9 and Arg94 that protrude from the base of the MR1 A′-pocket. Changing the terminal carboxylic acid group of DB28 for an ester in NV18.1 causes no major structural changes within the MR1 pocket between both complexes. Collectively, a pattern of intermolecular hydrophobic and polar interactions is formed between DB28/NV18.1 ligands and the MR1 A′-pocket that sequester the ligands inside the cleft. No direct or water-mediated contacts were observed between the DB28/NV18.1 ligands and any of the TCR CDR3 and CDRβ loops (Fig. 9I–K), in agreement with the inability of these compounds to bind to the MAIT TCR (Fig. 8) and the capacity of DB28 to activate MAIT cells (Fig. 6).

**Discussion**

During the past 7 y, several MAIT cell agonists and inhibitors have been identified (23, 24, 26). Stimulatory microbial ligands are characterized by the presence of a ribityl moiety, while their potential generally correlates with the ability of the Schiff base with Lys43 in the MR1 A′-pocket (38, 39). Formation of the Schiff base is thought to be the key molecular trigger for MR1 translocation to the cell surface, which is transiently observed upon ligand exposure (20). Through an in silico screen, we have identified additional MR1-binding ligands and now report the identity of a ligand that down-regulates MR1 cell surface expression. Unlike 5-OP-RU or Ac-6-FP, exposure to DB28 does not lead to MR1 translocation to the cell surface, and in primary monocytes, B cells, and both myeloid and B cell lines, it reduces MR1 basal levels of expression. Using epithelial cells expressing MR1 fused to GFP and intracellular staining for total MR1 proteins, we demonstrated that MR1 molecules are not degraded; rather, they are retained intracellularly in an EndoH-sensitive compartment, likely the ER/early Golgi. Consequently, DB28 is able to competitively inhibit MAIT cell activation by antigen-presenting cells pulsed with strong and weak synthetic agonists or infected with bacillus Calmette-Guérin or E. coli, bacteria that are both able to synthesize vitamin B2 metabolites that are strong MAIT cell agonists. Down-regulation of MR1 cell surface expression is observed with two monoclonal antibodies, recognizing different epitopes of correctly folded MR1 molecules, in agreement with the lack of complete maturation of MR1 molecules. The effect of DB28 is specific for MR1 molecules, as no down-regulation of MHC class I or CD1d molecules is observed. We also observed a trend of preferential inhibition of newly synthesized MR1 molecules, as demonstrated by lower MR1 down-regulation in the presence of the protein synthesis inhibitor cycloheximide or in cells expressing a doxycycline-inducible MR1 construct. This result suggests the existence of different compartments within the ER/early Golgi for distribution of MR1 ligands and might indicate differential association of MR1-loaded molecules with chaperones; for example, proteins of the peptide-loading complex. Thus, DB28-like molecules represent important tools for unraveling the molecular mechanisms of MR1-dependent antigen presentation. We investigated whether DB28 could be used in vivo to inhibit agonist-dependent MAIT cell activation. Despite the molar excess of DB28, we did not observe any inhibitory effect, likely because of the Schiff base with Lys43 in the MR1 A′-pocket. However, it is stabilized by a network of hydrophobic interactions and hydrogen bonds with the charged arginine residues. The lack of any inhibitory effect on Lys43Ala mutant MR1 molecules might be explained by the rapid egress of these molecules from the ER in the absence of any exogenous ligand (20). Other MR1 ligands

**Fig. 7.** DB28 reduces the stimulatory activity of agonist-pulsed MR1-expressing antigen-presenting cells. (A and B) THP1-MR1 were pulsed with the indicated concentrations of DB28 2 h before pulsing with DB19 (20 μg/mL) (A) or 5-OP-RU (5 ng/mL) (B) and addition of MAIT cells. In B, DB28 was used at 20 μg/mL IFN-γ (mean ± SD of technical duplicates) was measured in the supernatant collected after 16 h of stimulation. (C) MAIT cell stimulation by BEAS28 infected with bacillus Calmette-Guérin (BCG) at the indicated MOI, in the presence or absence of DB28 (20 μg/mL). Plotted is the percentage of response DB28/DMSO, where DMSO controls have been normalized to 100. n = 3 experimental replicates, each performed in technical duplicates. Raw data for one donor shown in SI Appendix, Fig. S9B. Multiple t test. *P ≤ 0.05; **P ≤ 0.005. (D) MAIT cell stimulation in PBMC infected with the indicated MOI of E. coli in the presence or absence of DB28 (20 μg/mL). Plotted is the percentage of response DB28/DMSO. n = 4 experimental replicates (2 for MOI 10). (Raw data for one donor shown in SI Appendix, Fig. S9 C and D.) Multiple t test. **P ≤ 0.005.

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(and weaker MAIT cell agonists) have been described that also lack the ability to form a Schiff base with Lys 43; these include ribityl lumazines (22, 23) and diclofenac (26). While our results confirm and extend the observation that MR1 molecules are loaded in the ER (20) and the need for a Schiff base to trigger MR1 release to the cell surface, they also suggest that weaker, non-Schiff base-forming ligands may be loaded on the cell surface or in the recycling compartment. The ester analog of DB28, molecules indicates that the extra methyl group in NV18.1 might impart more flexibility on the complex. Neither DB28 nor NV18.1, is less potent than DB28 in downregulating MR1 molecules in the tumor microenvironment.

In conclusion, we have identified a compound able to competitively down-regulate MR1 cell surface expression that may prove to be a useful tool compound for in vivo modulation of MAIT cell function. We have also identified additional MAIT cell agonists, which, similar to diclofenac (26), lack a ribityl moiety, and like lumazines (24), they are not predicted to form Schiff bases. Although weaker agonists than vitamin B2 intermediates, these compounds could be the starting point for structure-activity studies aimed at designing novel ligands that drive MAIT cell-dependent DC and B cell maturation (9, 44, 45).

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**Fig. 8.** Steady-state affinity measurements of MR1-Ag and MAIT TCRs. (A) SPR affinity measurements for MR1 presenting (A) 5-OP-RU, (B) Ac-6-FP, (C) DB28, and (D) NV18.1 compounds against two different MAIT TCRs: A-F7 (TRAV1-2-TRBV6-1) and TRAV1-2-TRBV6-4. SPR runs were conducted in duplicate, three independent experiments. The SPR sensorgrams (Middle), equilibrium curves (Right), and steady state $K_D$ values ($\mu$M) were prepared in Prism 7.
Materials and Methods

Medium and Reagents. The complete medium (CM) used throughout was RPMI 1640 (Gibco) for THP1 (ATCC), DMEM (Gibco) for BEAS2B, and IMDM (Gibco) for human MAIT cells. CM was supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% pen/strep, 5 × 10^{-5} M methylglyoxal (MG; Sigma) and 0.2% glycerol. The bacteria were frozen down at 1.34 × 10^{10} CFU/mL, and thawed aliquots were passaged 10 times through a 27G syringe before infection. DH5α was a gift from Peter Sander. Bacillus Calmette-Guérin (gift from Peter Sander) was grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC (Thermo Fisher Scientific) and 0.2% glycerol. The bacteria were frozen down at 1.34 × 10^{10} CFU/mL, and thawed aliquots were passaged 10 times through a 27G syringe before infection. DH5α E. coli bacteria (Thermo Fisher) were grown overnight to stationary phase in Luria broth, extensively washed in PBS, and diluted to an OD600 of 0.5 (equivalent to about 400 million bacteria/mL). Acetyl-6-FP was purchased from Schircks laboratories and was resuspended in DMSO at 2 mg/mL. 5-A-RU was synthesized as described in the supplementary methods and was combined with 50 μM methylglyoxal (MG; Sigma) before each assay to obtain 5-OP-RU. MG was used alongside DMSO as a negative control in each stimulation assay. DB28 was purchased from Vitaslab.com (product STK870291) and resuspended in DMSO at 10 mg/mL. Cycloheximide and doxycycline were purchased from Sigma and resuspended in 80 °C protected DMSO. All compounds were stored in small aliquots at –80 °C, protected from light.

Generation of MAIT Cells and Antigen-Presenting Cells. Blood was obtained from the UK National Blood Service. Human MAIT cells were isolated by cell sorting CD2 MACS enriched leukocytes with CD161 and Vα7.2 antibodies (Biolegend). In some experiments, antibodies to TRBV20.1 (Miltenyi) or TRBV13.2 (cone H132, Biolegend) were added to sort MAIT cell subsets. MAIT cells were grown for 3 wk in CM supplemented with IL-2. iNKT cells were generated and maintained as described (47). The MR1-restricted CD8+ T cell clone D426-G11 was previously described (48).

Jurkat expressing MAIT TCRs have been previously described (29). THP-1 MR1-HA cells were generated transducing THP-1 cells with a lentivirus encoding for MR1-HA tagged molecules, cloned in the lentiviral vectors pHR-SIN with the following primers: forward: taaccgAGATCTGCTGCACCACCTACGGGATCCGGGCAGCGTGCGCTGCTGTTGGAA; reverse: (5′)gcgaagACGTATCGTATGGGGAACATCGTATGGGTAtcgatctggtgttggaa.

Stimulation Assays. THP-1 cells were plated at 50,000 cells/well in 96-well U-bottom plates in CM and incubated with MAIT cells (20,000 cells/well, in triplicate) in the presence or absence of different concentrations of DB19, 5-OP-RU, and DB28. DB28 (20 μg/mL) was added 2 h before 5-OP-RU unless otherwise stated. MAIT cell activation was assessed by IFN-γ ELISA (antibodies to human IFN-γ, from R&D systems) and flow cytometry (BD FACSCanto II or BD LSR II).

Fig. 9. Ternary structures of AF-7-TCR-MR1 inhibitor complexes. (A) Overlay of the TCR-MR1 ternary structures of 5-OP-RU (PDB ID: 6PUC), DB28 and NV18.1 compounds. MR1 and Jαm are colored gray and pale cyan, respectively. TCR chains and the ligands are colored as follows: 5-OP-RU, green; DB28, orange; and NV18.1, wheat. (B) The top view of MR1 cleft with the CDRα, CDRβ) loops is shown as cartoon and colored as in A. (C and D) Omit maps contoured at 3σ (Fo –Fc; map; green mesh) of DB28 (C) and NV18.1 (D) after stimulated annealing refinements in Phenix crystallography package. (E) Superposition of DB28, NV18.1, and 5-OP-RU metabolites within the binding cleft. (F and G) Detailed interaction of DB28 (F) and NV18.1 (G) within the A-pocket of MR1 binding cleft. Intermolecular and intramolecular H-bonds are represented as black and deep-teal dashes, respectively. (H) Superposition of all pocket residues of DB28, NV18.1, and 5-OP-RU compounds highlighting small conformational differences of MR1 residues between various structures. The residues of MR1-DB28 and NV18.1 are colored light blue. The interacting residues of MR1-5-OP-RU are colored light pink in DB28 and NV18.1 structures, and lemon in 5-OP-RU complex.
E. coli and after extensive washing in PBS, OD

10474 machine and analyzed with FlowJo 10. Viability was assessed with live/dead in Brilliant violet buffer (BD) with the following antibodies: BUV661 CD3 (Infrared), according to the manufacturer the anti-HA mouse monoclonal antibody (clone 2

determined on fixed and permeabilized cells (Foxp3 kit; ThermoFisher) with surface MR1 (clone 26.5, Biolegend; clone 8F2F9, purified inhouse) or CD1d

5-OP-RU, or 1

with 5-OP-RU (10

propylene conical tubes (BD Falcon). One milliliter of blood was activated in BEAS2B Tet MR1-GFP Assays.

1. D. I. Godfrey, H. F. Koay, J. McCluskey, N. A. Gherardin, The biology and functional importance of MAIT cells. Nat. Immunol. 20, 1110–1128 (2019).

2. M. Salio, V. Cerundolo, Regulation of lipid specific and vitamin specific non-MHC restricted T cells by antigen presenting cells and their therapeutic potentials. Front. Immunol. 6, 388 (2015).

3. N. A. Gherardin et al., Diversity of T cells restricted by the MHC class I-related molecule MR1 facilitates differential antigen recognition. Immunity 44, 32-45 (2016).

4. R. Rearttargon et al., Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. J. Exp. Med. 210, 2305-2320 (2013).

5. C. Leu et al., Antimicrobial activity of mucosal-associated invariant T cells. Nat. Immunol. 11, 701-708 (2010).

6. M. C. Gold et al., Human mucosal associated invariant T cells detect bacterially infected cells. PLoS Biol. 8, e1000407 (2010).

7. A. Toubal, I. Nel, S. Letersman, A. Leshue, Mucosal-associated invariant T cells and disease. Nat. Rev. Immunol. 19, 643–657 (2019).

8. R. Lamichhane et al., TCR- or cytokine-activated CD8+ mucosal-associated invariant T cells are rapid polyfunctional effectors that can coordinate immune responses. Cell Rep. 28, 3061-3076.e5 (2019).

9. M. Salio et al., Activation of human mucosal-associated invariant T cells induces CDS1-dependent maturation of monocyte-derived and primary dendritic cells. J. Immunol. 199, 2628–2638 (2017).

10. P. T. Rudak, I. Choi, S. M. M. Haeryfar, MAIT cell-mediated cytotoxicity: Roles in host defense and therapeutic potentials in infectious diseases and cancer. J. Leukoc. Biol. 104, 473–486 (2018).

Statistical Analysis. Statistical analyses were performed with GraphPad Prism software, version 8. Comparisons were performed with t test, and differences with P < 0.05 were deemed significant.

MR1 Docking. Constraints imposed during docking included the presence of an aromatic ring at a distance suitable for aromatic interactions with Y7. Four hydrogen-bonding interactions were required out of the selected interactions formed by co-crystallized ligands in the complex structures used for the visual screening. Poses lacking aromatic stacking interactions with Y7 residue of MR1 were excluded. Out of the top-scoring poses, the selection was based on favorable interactions with MR1/TRBV1 and the presence of suboptimal contacts. In the case of compounds with acceptable poses, only the most favorable pose was included in the final selection.

Protein Production and SPR Measurements. Soluble A-F7 MAIT TCR (TRAV1-2-TRBV6-1), #6 (TRAV1-2-TRBV6-4) TCR, and human MR1-2m-Ag were refolded from inclusion bodies and purified as described (22, 25). All SPR measurements were conducted in duplicate (n = 2) on a Biacore 3000 instrument, as described previously (25, 37). For extended description, see SI Appendix, Supplemental Experimental Procedures.

Cryostatization, Structure Determination, and Refinement. A-F7 TCR was mixed with MR1-2m-Ag in 1:1 ratio, and ternary complex crystals were obtained by hanging drop crystallization, as established previously (22). Data were collected at the Australian Synchrotron Facility, processed and refined with standard software packages. For an extended description, see SI Appendix, Supplemental Experimental Procedures.

Accession Numbers. The coordinates of the ternary complexes of MAIT A-F7 TCR-MR1-B286 and TCR-MR1-11V181 have been deposited in the Protein Data Bank under accession codes 6PV6 and 6PVU.

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1. I. Magalhaes et al., Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. J. Clin. Invest. 125, 1752–1762 (2015).

2. D. Roussel et al., Cytotoxic and regulatory roles of mucosal-associated invariant T cells in type 1 diabetes. Nat. Immunol. 18, 1321-1331 (2017).

3. E. Bianchini et al., Invariant natural killer T cells and mucosal-associated invariant T cells in multiple sclerosis. Immunol. Lett. 183, 1–7 (2017).

4. N. E. Serriari et al., Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. Clin. Exp. Immunol. 176, 266-274 (2014).

5. T. Leing et al.; Oxford BD investigators, TCR and inflammatory signals tune human MAIT cells to exert specific tissue repair and effector functions. Cell Rep. 28, 3077–3091.e5 (2019).

6. T. C. S. Hinks et al., Activation and in vivo evolution of the MAIT cell transcriptome in mice and humans reveals tissue repair functionality. Cell Rep. 28, 3249–3262.e5 (2019).

7. M. Dasseaux et al., Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161+ IL-17-secreting T cells. Blood 117, 1250–1259 (2011).

8. A. Rahimpour et al., Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. J. Exp. Med. 212, 1095–1108 (2015).

9. N. L. Bouhour et al., MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. PLoS Pathog. 9, e1003681 (2013).

10. H. E. Williamson et al., The intracellular pathway for the presentation of vitamin B-related antigens by the antigen-presenting molecule MR1. Nat. Immunol. 17, 531–536 (2016).
21. W. Awad, J. Le Nours, L. Kjer-Nielsen, J. McCluskey, J. Rossjohn, Mucosal-associated invariant T cell receptor recognition of small molecules presented by MR1. *Immunol. Cell Biol.* **96**, 588–597 (2018).
22. G. Patel et al., Recognition of vitamin B metabolites by mucosal-associated invariant T cells. *Nat. Commun.* **4**, 2142 (2013).
23. L. Kjer-Nielsen et al., MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **481**, 717–723 (2012).
24. A. J. Corbett et al., T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361–365 (2014).
25. S. B. Eickle et al., A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells. *J. Exp. Med.* **211**, 1585–1600 (2014).
26. A. N. Keller et al., Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. *Nat. Immunol.* **18**, 402–411 (2017).
27. J. López-Sagaseta et al., MAIT recognition of a stimulatory bacterial antigen bound to MR1. *J. Immunol.* **191**, 5268–5277 (2013).
28. S. Huang et al., Evidence for MR1 antigen presentation to mucosal-associated invariant T cells. *J. Biol. Chem.* **280**, 21183–21193 (2005).
29. L. J. Howson et al., MAIT cell clonal expansion and TCR repertoire shaping in human volunteers challenged with Salmonella Paratyphi A. *Nat. Commun.* **9**, 253 (2018).
30. W. J. Chua et al., Endogenous MHC-related protein 1 is transiently expressed on the plasma membrane in a conformation that activates mucosal-associated invariant T cells. *J. Immunol.* **186**, 4744–4750 (2011).
31. M. J. Miley et al., Biochemical features of the MHC-related protein 1 consistent with an immunological function. *J. Immunol.* **160**, 6090–6098 (2003).
32. P. Riegert, V. Wanner, S. Bahram, Genomics, isoforms, expression, and phylogeny of the MHC class I-related MR1 gene. *J. Immunol.* **161**, 4066–4077 (1998).
33. E. Treiner et al., Mucosal-associated invariant T (MAIT) cells: An evolutionarily conserved T cell subset. *Microbes Infect.* **7**, 552–559 (2005).
34. E. Karamooz, M. J. Harriff, G. A. Narayanan, A. Worley, D. M. Levinsohn, MR1 recycling and blockade of endosomal trafficking reveal distinguishable antigen presentation pathways between Mycobacterium tuberculosis infection and exogenously delivered antigens. *Sci. Rep.* **9**, 4797 (2019).
35. G. A. Narayanan et al., Alternative splicing of MR1 regulates antigen presentation to MAIT cells. bioRxiv:10.1101/695296 (11 July 2019).
36. B. Laugel et al., Engineering of isogenic cells deficient for MR1 with a CRISPR/Cas9 lentiviral system: Tools to study microbial antigen processing and presentation to human MR1-restricted T cells. *J. Immunol.* **197**, 971–982 (2016).
37. W. Awad et al., The molecular basis underpinning the potency and specificity of MAIT cell antigens. *Nat. Immunol.* **21**, 400–411 (2020).
38. H. E. McWilliam, R. W. Birkinshaw, J. A. Villadangos, J. McCluskey, J. Rossjohn, MR1 presentation of vitamin B-based metabolite ligands. *Curr. Opin. Immunol.* **34**, 28–34 (2015).
39. A. N. Keller, A. J. Corbett, J. M. Wubben, J. McCluskey, J. Rossjohn, MAIT cells and MR1-antigen recognition. *Curr. Opin. Immunol.* **46**, 66–74 (2017).
40. L. Van Kaer, NKT cells: T lymphocytes with innate effector functions. *Curr. Opin. Immunol.* **19**, 354–364 (2007).
41. K. S. Forsythe, L. C. Eisenlohr, Giving CD4+ T cells the slip: Viral interference with MHC class II-restricted antigen processing and presentation. *Curr. Opin. Immunol.* **40**, 123–129 (2016).
42. A. B. Schuren, A. I. Costa, E. J. Wiertz, Recent advances in viral evasion of the MHC class I processing pathway. *Curr. Opin. Immunol.* **40**, 43–50 (2016).
43. E. W. Meemeier, M. J. Harriff, E. Karamooz, D. M. Levinsohn, MAIT cells and microbial immunity. *Immunol. Cell Biol.* **96**, 607–617 (2018).
44. M. S. Bennett, S. Trivedi, A. S. Iyer, J. S. Hale, D. T. Leung, Human mucosal-associated invariant T (MAIT) cells possess capacity for B cell help. *J. Leukoc. Biol.* **102**, 1261–1269 (2017).
45. M. Lepore et al., Functionally diverse human T cells recognize non-microbial antigens presented by MR1. *eLife* **6**, e24476 (2017). Correction in: *eLife* **6**, e29743 (2017).
46. M. K. Wild et al., Dependence of T cell antigen recognition on the dimensions of an accessory receptor-ligand complex. *J. Exp. Med.* **190**, 31–41 (1999).
47. M. Salio et al., Saposins modulate human invariant natural killer T cells self-reactivity and facilitate lipid exchange with CD1d molecules during antigen presentation. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e4753–e4761 (2013).
48. M. C. Gold et al., MR1-restricted MAIT cells display ligand discrimination and pathogen selectivity through distinct T cell receptor usage. *J. Exp. Med.* **211**, 1601–1610 (2014).