MR1-restricted MAIT cells display ligand discrimination and pathogen selectivity through distinct T cell receptor usage

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Mucosal-associated invariant T (MAIT) cells express a semi-invariant T cell receptor (TCR) that detects microbial metabolites presented by the nonpolymorphic major histocompatibility complex (MHC)–like molecule MR1. The highly conserved nature of MR1 in conjunction with biased MAIT TCRα chain usage is widely thought to indicate limited ligand presentation and discrimination within a pattern–like recognition system. Here, we evaluated the TCR repertoire of MAIT cells responsive to three classes of microbes. Substantial diversity and heterogeneity were apparent across the functional MAIT cell repertoire as a whole, especially for TCRβ chain sequences. Moreover, different pathogen–specific responses were characterized by distinct TCR usage, both between and within individuals, suggesting that MAIT cell adaptation was a direct consequence of exposure to various exogenous MR1–restricted epitopes. In line with this interpretation, MAIT cell clones with distinct TCRs responded differentially to a riboflavin metabolite. These results suggest that MAIT cells can discriminate between pathogen–derived ligands in a clonotype–dependent manner, providing a basis for adaptive memory via recruitment of specific repertoires shaped by microbial exposure.

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It is established that MAIT cells detect a broad range of microorganisms (Gold et al., 2010; Le Bourhis et al., 2010) and contribute to the control of several pathogens in mice, including *Klebsiella pneumoniae* (Georgel et al., 2011), *Francisella tularensis* (Meierovics et al., 2013), and mycobacterial species (Le Bourhis et al., 2010; Chua et al., 2012). Furthermore, MAIT cells expand in the periphery after thymic egress as a consequence of microbial colonization (Martin et al., 2009; Le Bourhis et al., 2010). In line with these observations, human MAIT cells display a naïve phenotype in cord blood but acquire characteristics associated with exogenous antigen exposure in adults (Dusseaux et al., 2011; Gold et al., 2013). However, the microbial and environmental signals that lead to the migration, differentiation, expansion, and maintenance of MAIT cells are not understood.

Although the MAIT TCR has been described as semi-invariant, there is sufficient plasticity within the repertoire to allow for the possibility of diverse ligand recognition. Indeed, MAIT TCRs are not exclusively germline-encoded and incorporate sequence variability as a consequence of V(D)J recombination (Tilloy et al., 1999; Greenaway et al., 2013; Reantragoon et al., 2013). This process of somatic gene rearrangement potentially enables pathogen-specific recognition within the confines of restricted TCR gene usage.

Recently, MR1 has been shown to bind small organic compounds derived from microbial vitamin B biosynthetic pathways (Kjer-Nielsen et al., 2012). Although MR1 binds metabolites of vitamin B9 (folic acid), only three derivatives of vitamin B2 (riboflavin), rRL-6-CH2OH, RL-6-Me-7-OH, and RL-6,7-diMe, are known to stimulate MAIT cells (Kjer-Nielsen et al., 2012). Consistent with this observation, microorganisms with the capacity to synthesize riboflavin have been associated with MAIT cell activation (Gold et al., 2010; Le Bourhis et al., 2010; Kjer-Nielsen et al., 2012). However, structural studies have revealed that MR1 is promiscuous in its ability to bind and present diverse ligands (Reantragoon et al., 2012; López-Sagasta et al., 2013a; Patel et al., 2013). Accordingly, there may be discrete pathogen-associated ligands that are yet to be identified.

In this study, we evaluated the ex vivo TCR repertoire of pathogen-reactive MAIT cells responsive to *Mycobacterium smegmatis*, *Salmonella typhimurium*, and *Candida albicans*. Clone type usage was complex and highly selective within individuals, suggesting a diverse array of MR1-restricted microbial ligands. Moreover, different pathogen-specific responses were characterized by distinct TCR repertoires, both between and within individuals. Direct evidence of MR1-restricted ligand discrimination at the clonal level further supported the potential for unique patterns of pathogen recognition by MAIT cells. Collectively, these data provide a basis for specific TCR repertoire mobilization within an innate-like system shaped by previous microbial exposure.

### RESULTS

The TCR repertoire of MAIT cells identified using an MR1 tetramer loaded with the stimulatory vitamin B metabolite, rRL-6-CH2OH, was recently shown to be more heterogeneous than expected based on previous studies (Reantragoon et al., 2013). Moreover, MAIT cells can detect infection with a wide range of bacteria and fungi (Gold et al., 2010; Le Bourhis et al., 2010), which likely have diverse metabolic pathways. In this context, we aimed to determine the relationship between TCR usage and pathogen discrimination.

In earlier work, we used limiting dilution analysis to generate a panel of *Mycobacterium tuberculosis* (Mtbt–reactive CD8*+* MAIT cell clones (Gold et al., 2010). All of these clones expressed TRAV1-2–TRAJ33 TCRs and recognized Mtbt–infected cells in an MR1-restricted manner as determined by Vα7.2–specific mAb staining and functional blockade with an αMR1 mAb, respectively (not depicted; Gold et al., 2010). Sequence analysis confirmed this finding and further revealed that the majority of clones also incorporated the canonical TRAJ33 gene segment (Table 1). However, 4/16 clones expressed TRAJ20 (Gold et al., 2010), suggesting that pathogen-reactive MAIT cells could yield additional TCR specificities. Moreover, we observed that MAIT cells from different individuals segregated into TCR families through preferential pairing of specific TCRα and TCRβ chains (Table 1). For example, clones A9 and F9–2 from donors 481 and 504, respectively, expressed identical TRAV1-2/TRAJ33*α* chains paired with TRBV20-1*β* chains.

To extend these findings beyond the Mtbt–specific setting, we used an unbiased molecular approach to evaluate the MAIT TCR repertoire across diverse bacterial and fungal species. Peripheral blood CD8*+* T cells from four unrelated adult donors were stimulated directly ex vivo by exposure to A549 epithelial cells infected with *M. smegmatis*, *S. typhimurium*, or *C. albicans* to identify pathogen–reactive MAIT cells restricted by MR1 (Gold et al., 2010, 2013). Functionally responsive MAIT cells display pathogen selectivity | Gold et al.
Identified. Of these noncanonical genes, TRAJ9 and TRAJ39 do not encode the Tyr95 residue present in TRAJ33, TRAJ20, and TRAJ12 (Reantragoon et al., 2013), which is thought to be critical for MAIT cell activation (Reantragoon et al., 2012; Patel et al., 2013; Young et al., 2013). Due to the lack of allelic exclusion at the TRA locus, however, we cannot definitively assign these \( \alpha \) chains to functional heterodimeric TCRs.

Notable differences in TRAJ gene usage were apparent across pathogen-reactive MAIT cell populations. In terms of overall distribution, diversity was greatest for \textit{S. typhimurium} and most restricted for \textit{C. albicans}. Individual TRAJ gene usage also differed across specificities within each individual. This was most striking in donor 520 (D520). Here, TRAV1-2/TRAJ33 gene–encoded TCRs were elicited in response to \textit{M. smegmatis} and \textit{S. typhimurium}, but not in response to \textit{C. albicans}. Collectively, these data demonstrate that pathogen-reactive TRAV1-2+ MAIT cells can productively incorporate a broad array of TRAJ gene segments with differential usage across pathogen specificities.

Cells were isolated for repertoire experiments at high purity using a flow cytometric sorting strategy to detect viable TRAV1-2+ cells expressing TNF (Haney et al., 2011). Response sizes varied from 0.5 to 6.9% of the TRAV1-2+ CD8+ T cell population with no significant differences between pathogens. Clonotypic analysis was then performed using a template switch–anchored RT-PCR as described previously (Quigley et al., 2011).

An aggregated analysis of TRAJ gene usage by pathogen-reactive TRAV1-2+ MAIT TCRs is shown in Fig. 1. Although the canonical TRAV1-2/TRAJ33 rearrangement dominated the TCR\( \alpha \) chain repertoire, alternative TRAJ genes were commonly used. In particular, substantial proportions of the TRAV1-2/non-TRAJ33 rearrangements incorporated TRAJ20 or TRAJ12 segments. Moreover, an array of TRAJ genes not previously known to associate with pathogen-reactive, TRAV1-2+ MAIT cells were identified. Of these noncanonical genes, TRAJ9 and TRAJ39 do not encode the Tyr95\( \alpha \) residue present in TRAJ33, TRAJ20, and TRAJ12 (Reantragoon et al., 2013), which is thought to be critical for MAIT cell activation (Reantragoon et al., 2012; Patel et al., 2013; Young et al., 2013). Due to the lack of allelic exclusion at the TRA locus, however, we cannot definitively assign these \( \alpha \) chains to functional heterodimeric TCRs.

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A similar analysis is shown for TRBV gene usage in Fig. 2. Across the dataset as a whole, 35 different TRBV genes were represented within the pathogen-reactive MAIT cell repertoire. Most frequent among these were the TRBV20-1 and TRBV6 family gene members. In contrast, these TRBV genes were used by <6% of nonresponsive TRAV1-2+ cells, which expressed a diverse array of TRBV genes without apparent enrichments (not depicted). As above, TRBV gene usage was most diverse for S. typhimurium and varied across specificities within each individual. In D520, for example, the MAIT cell response to C. albicans was composed entirely of TRBV20-1+ TCRs, yet these were relatively rare in the corresponding responses.

Figure 3. MAIT TRAV1-2+ CDR3α sequences display length and sequence similarity. (A) Representation of unique TRAV1-2+ CDR3α sequences from each donor in response to each pathogen as indicated. Each bar represents a distinct sequence, the relative frequency of which is depicted on the y axis. TRAJ gene usage is shown on the x axis labeled for dominant sequences. (B) Distribution of CDR3α amino acid lengths from TRAV1-2+ MAIT cells responsive to each pathogen as indicated in the key. (C) Visual representation of amino acid enrichments at each position across the CDR3α core compiled from pathogen-reactive MAIT cell TRAV1-2+ sequences. Analysis was confined to CDR3α sequences with a length of 11 amino acids, encompassing 39 sequences for M. smegmatis, 61 for S. typhimurium, and 30 for C. albicans. Graphics were generated using Seq2Logo.
to *S. typhimurium* and *M. smegmatis*. Thus, MAIT cells use diverse TRBV gene segments with variable clonotypic deployment across pathogen specificities.

Previous studies have demonstrated that MAIT TCRα chains are not exclusively germline encoded (Tilloy et al., 1999; Treiner et al., 2005; Gold et al., 2010; Goldfinch et al., 2010) and most often contain single nucleotide additions that contribute to CDR3 core diversity (Greenaway et al., 2013). We therefore performed an in-depth analysis of CDR3α sequences across our dataset (Fig. 3).

Unique CDR3α sequences within the TRAV1-2+ repertoire are displayed across donors and specificities in Fig. 3 A. In these graphics, each bar represents a distinct sequence plotted as a function of TRAJ gene usage (x axis) and relative frequency (y axis). Disparate patterns were observed, suggesting pathogen-specific MAIT cell reactivity. In D497, for example, *M. smegmatis* and *C. albicans* elicited three predominant TRAV1-2+ sequences that were distinct from one another; a broader repertoire was activated in response to *S. typhimurium*, which again differed at the level of CDR3α sequence usage. Similarly, the TRAV1-2+ MAIT cell response to *C. albicans* was dominated by two unique sequences in D520, whereas broader repertoires were elicited by *M. smegmatis* and *S. typhimurium*. Thus, the MAIT cell response to each of these microbes was characterized by unique TCRα usage within individuals.

Although no unique TRAV1-2+ sequence appeared to associate with a single pathogen or a single donor, we wanted to evaluate patterns of amino acid usage across pathogen specificities. To perform this analysis on the greatest number of TRAV1-2+ transcripts, we selected CDR3α sequences containing 11 amino acid residues (Fig. 3 B). This subset contained 39 sequences for *M. smegmatis*, 61 for *S. typhimurium*, and 30 for *C. albicans*. Representative logos highlighting preferential amino acid usage for each pathogen are shown in Fig. 3 C. Particular amino acid motifs were prevalent within the TRAV1-2+ sequences for each pathogen. For example, tyrosine (Y) was highly prevalent at position 8 in *C. albicans*-reactive MAIT cells, whereas glycine (G) was favored at position 7 for *S. typhimurium*. To quantify this observation further, we compared the similarity of TRAV1-2+ sequences within a given group, defined by donor or pathogen, to the corresponding sequence similarity between groups (Shen et al., 2012). The analysis across donors shown in Table 2 (A) demonstrates two points. First, overall similarity is high, given that a value of 1 represents 100% similarity. Second, similarity within each donor is consistently greater than the similarity between donors. Similar patterns were observed across pathogen specificities (Table 2, B). The significance of these analyses was evaluated using an exact permutation test. Here, each observed TRAV1-2+ sequence was randomly assigned to a donor-pathogen pair to determine whether such associations could lead to a result where the similarity within donors and pathogens (intrasimilarity) was consistently higher than the similarity between donors and pathogens (intersimilarity). None of the permutations across 1,000 rounds resulted in higher intersimilarity (*P* < 0.001). Collectively, these data suggest that MAIT TCRα chains can discriminate between ligands. Although we have no knowledge of each individual's microbial exposure history, the repertoire observed for any particular microbial pathogen may reflect prior ligand exposure. At present, the precise nature of such MR1-restricted antigens and their relationship to TCR usage remains unknown.

To assess true clonotypic diversity, we similarly evaluated TRBV chain usage across donors and specificities (Fig. 4). For this purpose, we selected TRBV20-1+ sequences, which represent the most common TRBV gene–defined clonotypes across pathogen–reactive MAIT cells. As above, unique TRBV20-1+ sequences were associated with each pathogen specificity. In D497, for example, MAIT cell populations specific for *S. typhimurium* and *C. albicans* incorporated different CDR3β loops despite equivalent TRBV20-1 gene usage (Fig. 4 A); these sequences were further distinct from those responsive to *M. smegmatis* (Fig. 4 A). Similar findings applied to other TRBV gene families (not depicted). Moreover, across the entire dataset, only four shared TCRβ clonotypes were detected between donors; notably, each of these sequences was associated with more than one pathogen specificity (not depicted). Thus, in sharp contrast to the TCRα chain repertoire, minimal TCRβ overlap was present across donors and pathogens.

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**Table 2.** Similarity analyses of TRAV1-2+ CDR3 sequences

Bolded values denote those with the highest similarity within each comparison group.
MAIT cells display pathogen selectivity | Gold et al.

To evaluate amino acid usage across specificities, we selected all clonotypes with CDR3β sequences containing 13 or 14 residues, based on prevalence within the length distribution profile (Fig. 4 B). This subset contained 34 sequences for M. smegmatis, 75 for S. typhimurium, and 39 for C. albicans. No obvious motifs were present across the CDR3β core, although TRBJ gene–encoded phenylalanine (F) and tyrosine (Y) residues were notably represented at the C terminus, potentially reflecting amino acid conservation linked to preferential usage of TRBJ1-1, TRBJ2-1, and TRBJ2-7 (Fig. 4 C). To extend these observations, we performed similarity analyses for the most common TRBV gene–defined clonotypes in our dataset.

Figure 4. MAIT CDR3β usage is unique across individuals and pathogens. (A) Representation of unique TRBV20-1+ CDR3β sequences from each donor in response to each pathogen as indicated. Each bar represents a distinct sequence, the relative frequency of which is depicted on the y axis. TRBJ gene usage is shown on the x axis labeled for dominant sequences. (B) Distribution of CDR3β amino acid lengths from all TRBV gene–defined MAIT cells responsive to each pathogen as indicated in the key. (C) Visual representation of amino acid enrichments at each position across the CDR3β core compiled from pathogen-reactive MAIT cell TRBV+ sequences. Analysis was confined to CDR3β sequences with a length of 13 or 14 amino acids, encompassing 34 sequences for M. smegmatis, 75 for S. typhimurium, and 39 for C. albicans. Graphics were generated using Seq2Logo.
(TRBV20-1, TRBV6-1, TRBV6-2/3, and TRBV28), comprising a total of 148 unique sequences across all donors and pathogens (Table 3, A and B). Again, the similarity indices were consistently high, but nevertheless lower than for the TRAV1-2+ sequences, reflecting a more diverse TCRβ repertoire. Furthermore, intrasimilarity was significantly greater than intersimilarity for all TRBV gene–defined comparison groups across donors (Table 3, A) and pathogens (Table 3, B). This latter observation is particularly remarkable in the case of S. typhimurium, which mobilized a highly diverse set of TCRβ clonotypes (median Simpson's diversity index, 0.934–0.983) compared with M. smegmatis and C. albicans (median Simpson's diversity index, 0.221/0.184 to 0.971/0.966, respectively). Finally, we compared the similarity of TRBV gene–defined sequences in TRAV1-2+ nonreactive (TNF−) cells isolated during the C. albicans stimulation protocol with the corresponding pathogen-reactive (TNF+) populations. The greatest level of similarity was observed among the nonreactive TRAV1-2+ cells (Table 3, C). Collectively, these analyses support the conclusion that different pathogen-associated antigens drive distinct donor-specific TCRβ clonotype expansions within the MAIT cell population.

To confirm this hypothesis, we tested four TCR–distinct MAIT cell clones (Table 1; Gold et al., 2010) for reactivity against the riboflavin-derived MR1-restricted ligand RL-6,7-diMe (Kjer-Nielsen et al., 2012) in IFN-γ ELISPOT assays. Only two clones recognized RL-6,7-diMe (Fig. 5 A), even though all four were comparably stimulated by presenting cells infected with M. smegmatis. The stronger agonist rRL-6-CH2OH was not tested (Kjer-Nielsen et al., 2012; Corbett et al., 2014). Moreover, each clone responded similarly to each of the three pathogens used for TCR analysis (Fig. 5 B). Notably, all four clones contain the Tyr95 residue thought to be essential for MR1-restricted recognition (Reantragoon et al., 2012; Patel et al., 2013). Our study identifies two additional TRAV genes, namely TRAV13 and TRAV22, which also encode Tyr95 in MAIT cells. Further TRAV gene transcripts, (Porcelli et al., 1993; Tilloy et al., 1999). Additional TCRα complexity was subsequently demonstrated in Mtb-reactive MAIT cell clones (Gold et al., 2010) and more recently in rRL-6-CH2OH/MR1 tetramer–reactive cells (Reantragoon et al., 2013). In line with the latter study, pathogen-reactive MAIT cells frequently expressed TRAJ33, TRAJ32, and TRAJ20 gene transcripts. Each of these genes encodes the Tyr95 residue thought to be essential for MR1-restricted ligand recognition (Reantragoon et al., 2012; Lopez-Sagasta et al., 2013a; Patel et al., 2013). Our study identifies two additional TRAJ genes, namely TRAJ1 and TRAJ28, which also encode Tyr95 in MAIT cells. Further TRAJ gene transcripts,unreadable characters

**Table 3.** Similarity analyses of TRBV6-1+, TRBV6-2/3+, TRBV20-1+, and TRBV28+ CDR3 sequences

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(A) Similarity analyses across individuals

(B) Similarity analyses across pathogens

C. albicans

C. albicans

S. typhimurium

M. smegmatis

S. typhimurium

M. smegmatis

S. typhimurium

M. smegmatis

S. typhimurium

M. smegmatis

(S) Similarity analyses across nonreactive (TNF−) and pathogen-reactive (TNF+) subsets

TRAV1-2+ TNF−

M. smegmatis

C. albicans

S. typhimurium

C. albicans

M. smegmatis

M. smegmatis

M. smegmatis

TRAV1-2+ TNF−

TRAV1-2+ TNF−

TRAV1-2+ TNF−

TRAV1-2+ TNF−

Bolded values denote those with the highest similarity within each comparison group.

**DISCUSSION**

In this study, we evaluated the ex vivo TCR repertoire of MAIT cells responsive to three distinct classes of pathogens. Substantial diversity and heterogeneity were apparent across the functional MAIT cell repertoire as a whole, especially for TCRβ chain sequences. Moreover, different pathogen-specific responses were characterized by distinct TCR usage, both between and within individuals, suggesting that MAIT cell adaptation was a direct consequence of exposure to various exogenous MR1-restricted epitopes. In line with this interpretation, MAIT cell clones with distinct TCRs responded differentially to a riboflavin metabolite. These results suggest that MAIT cells can discriminate between pathogen-derived ligands in a clonotype-dependent manner, potentially enabling adaptive memory within a pattern-like recognition system.

MAIT cells were originally defined by the expression of a canonical semi-invariant TRAV1-2/TRAJ33 TCRα chain (Porcelli et al., 1993; Tilloy et al., 1999). Additional TCRα complexity was subsequently demonstrated in Mtb-reactive MAIT cell clones (Gold et al., 2010) and more recently in rRL-6-CH2OH/MR1 tetramer–reactive cells (Reantragoon et al., 2013). In line with the latter study, pathogen-reactive MAIT cells frequently expressed TRAJ33, TRAJ32, and TRAJ20 gene transcripts. Each of these genes encodes the Tyr95α residue thought to be essential for MR1-restricted ligand recognition (Reantragoon et al., 2012; Lopez-Sagasta et al., 2013a; Patel et al., 2013). Our study identifies two additional TRAJ genes, namely TRAJ1 and TRAJ28, which also encode Tyr95α in MAIT cells. Further TRAJ gene transcripts,
such as TRAJ9 and TRAJ39, were detected that do not encode Tyr95α. In one donor (DS20), these two genes dominated TRAJ usage in MAIT cells responsive to C. albicans, but not M. smegmatis or S. typhimurium, suggesting alternative modes of TCRα engagement. However, the lack of allelic exclusion at the TRA4 locus precludes definitive conclusions. Nonetheless, the TCRα repertoire of MAIT cells is clearly more diverse than previously described and shows features consistent with pathogen selectivity.

Historically, MAIT cells have been associated with TRBV20-1 and TRBV6 family genes (Porcelli et al., 1993; Tilloy et al., 1999). More recently, ~75% of tetramer-reactive MAIT cells were found to express these TRBV genes despite the presence of additional heterogeneity (Reantragoon et al., 2013). Although pathogen-reactive MAIT cells frequently displayed TRBV20-1 and TRBV6 family gene usage in our study, >50% of all TRBV family genes were also detected, revealing greater heterogeneity than observed previously. These findings could indicate promiscuous pairing or the potential for diverse ligand recognition mediated by the TCRβ chain. The extent to which the TCRVβ domain contributes to ligand discrimination remains unclear. Mutagenesis of specific residues in the CDR3β loop did not alter MAIT hybridoma cell recognition of MR1-overexpressing cells loaded with Salmonella supernatants; however, TCRVβ chain swapping abrogated recognition by the same hybridoma (Reantragoon et al., 2012). These data suggest that the TCRVβ domain contributes to MR1-restricted ligand recognition in ways that remain to be defined. At present, our ex vivo analysis of pathogen-reactive MAIT cells precludes the assignment of αβ pairing. Nonetheless, limited analysis of Mtb-reactive clones points to segregation of MAIT cells into TCR families through preferential pairing of specific TCRα and TCRβ chains. For example, MAIT cell clones with identical CDR3α chains isolated from different individuals tended to associate with the same TRBV gene family. Such putative TCR families may, at least in theory, contribute to greater affinities for specific classes of antigen. Indeed, the observation that only four TCRVβ sequences were shared across our entire dataset suggests that clonotypic expansions occur in a manner similar to conventional T cells. Accordingly, the observation of unique TRBV gene usage across pathogens suggests that TCRβ chains contribute to ligand detection. This conclusion is supported by molecular analyses of MAIT TCR engagement with MR1-presented ligands (Reantragoon et al., 2012; López-Sagaseta et al., 2013a,b; Patel et al., 2013; Young et al., 2013), which have revealed a docking mode similar to that of classical MHC-restricted αβ TCRs and unlike that used by CD1-restricted iNKT cells (Reantragoon et al., 2012; López-Sagaseta et al., 2013a,b; Patel et al., 2013). In this case, the less variable TCRα chain primarily mediates contacts with the MR1 protein, whereas the CDR3β loop is positioned above the MR1 ligand-binding groove. As suggested previously (López-Sagaseta et al., 2013a,b), this topography lends itself to the interpretation that the CDR3β loop can contribute to ligand discrimination.

Based on our findings, we postulate that MR1-restricted ligand diversity drives MAIT cell adaptation and TCR repertoire diversity. Although not yet described, we presume that the naïve MAIT thymocyte population contains a diverse and un-focused precursor repertoire. Subsequently, skewed clonotypic
responses to different pathogens emerge in the periphery as a result of antigenic exposure to a diverse array of MR1-restricted ligands. We further propose that these expansions will reflect distinct microbial challenges. This scenario of differential agonist encounter would explain the unique clonotypic expansions across individuals with different microbial exposure histories. In line with this hypothesis, increased MAIT TCR engagement through the CDR3β loop was recently associated with greater MR1-restricted ligand potency (López-Sagasta et al., 2013b; Patel et al., 2013). Thus, patterns of clonotypic expansion are likely to reflect high-affinity TCR interactions with distinct MR1 ligands, consistent with previous observations in conventional systems (Price et al., 2005).

Although the possibility exists that stimulatory microorganisms express the same few MR1-restricted riboflavin ligands in different proportions, elegant molecular analyses provide evidence that MR1 can accommodate a range of different ligands (Reantragoon et al., 2012; López-Sagasta et al., 2013a; Patel et al., 2013). To date, only three MAIT cell epitopes have been identified, all of which are riboflavin metabolites (Kjer-Nielsen et al., 2012; Reantragoon et al., 2012, 2013; Patel et al., 2013). Nonetheless, these antigens share many chemical properties with other microbial and mammalian compounds. It is therefore highly feasible that other MR1-restricted ligands are generated similarly, enabling MAIT cell adaptation to a wide range of microorganisms and their associated metabolomes.

Pathogen-reactive MAIT cells share many features traditionally thought to be associated with both innate and adaptive T cells. Restriction by a nonpolymorphic MHC-like molecule, expression of a semi-invariant TCR, thymically acquired effector function, and intrathymic selection by hematopoietic cells are all considered features of innate T cells. However, after thymic egress, MAIT cells expand and acquire an effector phenotype associated with antigenic exposure. Furthermore, the development of MAIT cells requires the presence of bacteria, suggesting the capacity to adapt in response to environmental signals. Our data provide evidence that MAIT cells can detect a diverse array of MR1-restricted ligands and differentiate selectively in response to specific pathogens akin to classically restricted T cells. Thus, the MAIT TCR enables adaptive immune responses within the confines of a pattern-like recognition system that can discriminate between microorganisms.

Microorganisms and infection of A549 cells. Adherent A549 cells were infected overnight with M. smegmatis (multiplicity of infection [MOI] 10), and nonadherent A549 cells were infected for 1 h with S. typhimurium (MOI 30) or C. albicans (MOI 3) at 37°C. All infections were performed in the absence of antibiotics.

Stimulation and isolation of pathogen-reactive MAIT cells. CD8+ T cells were enriched from PBMCs by negative selection using magnetic bead separation according to the manufacturer's instructions (STEMCELL Technologies), added to uninfected or infected A549 cells at a ratio of 3:1, and incubated for 16 h in the presence of α-TNF (Beckman Coulter) and TAP1-O (EMD Millipore) to prevent TNF cleavage from the cell surface (Hansey et al., 2011). The following day, cells were stained with: (a) α-CD4 and α-CD8 (BioLegend) and (b) α-TRAV1-2 and α-TCRγδ (Thermo Fisher Scientific). Dead cells were excluded from the analysis using propidium iodide (BioLegend). Viable CD4+ TCRγδ+CD8+TRAV1-2+TNF+ cells were sorted at high purity using a FACSAria flow cytometer (BD) directly into RNA-later (Applied Biosystems). The corresponding nonfunctional populations were sorted in parallel for control purposes. In all cases, cell numbers were standardized (5,000 per aliquot) to minimize sampling bias.

Molecular analysis of TCR usage. Clonotypic analysis of sorted cell populations was performed as described previously (Quigley et al., 2011). In brief, unbiased amplification of all expressed TRR or TRA gene products was conducted using a template switch–anchored RT-PCR with chain-specific constant region primers. Amplicons were subcloned, sampled, sequenced, and analyzed as described previously (Price et al., 2005). The IMGT nomenclature is used in this study (Lefranc et al., 2003).

Construction of sequence logos. Sequence logos were generated in Shannon format without the use of pseudo counts via the Seq2Logo web server (Thomson and Nielsen, 2012). Shannon sequence logos give a visual representation of amino acid enrichments at different positions in the observed CDR3 sequences. The different amino acids are colored according to physicochemical properties (acidic [DE]; red; basic [HKR]; blue; hydrophobic [ACFILMPVW]; black; and neutral [GNQSTY], green). For TRAV1-2, only sequences with a length of 11 amino acids were included (39 sequences for M. smegmatis, 61 for S. typhimurium, and 50 for C. albicans). For TRBV, only sequences with a length of 13 or 14 amino acids were included (34 sequences for M. smegmatis, 75 for S. typhimurium, and 39 for C. albicans). The TRBV sequences were aligned using ClustalX (Larkin et al., 2007) with default parameters set before logo generation. The width of each bar in the sequence logo corresponds inversely with gap density at the given positions.

CDR3 sequence similarity. Similarity between CDR3 sequences was calculated as described previously (Shen et al., 2012). This method allows similarities to be assigned between sequences of different length in an alignment-free manner. In this similarity measure, a perfect match has a similarity score of 1, and a perfect mismatch has a similarity score of 0. To calculate the similarity between two groups of sequences, each sequence from the first group was assigned a score corresponding to the best match against all sequences from the other group (excluding identical matches), and the similarity between the two groups was calculated as the mean of these similarity measures over all sequences within the first group.

CDR3 sequence diversity. Simpson’s diversity index was calculated using the randomization method as described previously (Venturi et al., 2007). The median value for each population was derived from 10,000 random draws standardized to the lowest sample size.

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