TCR Bias and Affinity Define Two Compartments of the CD1b–Glycolipid-Specific T Cell Repertoire

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TCR Bias and Affinity Define Two Compartments of the CD1b–Glycolipid-Specific T Cell Repertoire

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Current views emphasize TCR diversity as a key feature that differentiates the group 1 (CD1a, CD1b, CD1c) and group 2 (CD1d) CD1 systems. Whereas TCR sequence motifs define CD1d-reactive NKT cells, the available data do not allow a TCR-based organization of the group 1 CD1 repertoire. The observed TCR diversity might result from donor-to-donor differences in TCR repertoire, as seen for MHC-restricted T cells. Alternatively, diversity might result from differing CD1 isoforms, Ags, and methods used to identify TCRs. Using CD1b tetramers to isolate clones recognizing the same glycolipid, we identified a previously unknown pattern of V gene usage (TRAV17, TRBV4-1) among unrelated human subjects. These TCRs are distinct from those present on NKT cells and germline-encoded mycolipid–reactive T cells. Instead, they resemble the TCR of LDN5, one of the first known CD1b-reactive clones that was previously thought to illustrate the diversity of the TCR repertoire. Interdonor TCR conservation was observed in vitro and ex vivo, identifying LDN5-like T cells as a distinct T cell type. These data support TCR-based organization of the CD1b repertoire, which consists of at least two compartments that differ in TCR sequence motifs, affinity, and coreceptor expression. The Journal of Immunology, 2014, 192: 4054–4060.

Group 1 CD1 proteins (CD1a, CD1b, and CD1c) are thought to play a role in immunity because they present bacterial lipid Ags to human T cells. The identification of lipid Ags and cellular pathways of lipid Ag presentation, as well as proof of principle that CD1 and lipid-specific T cells can perform antimicrobial functions, was made possible by a small panel of T cell clones (1–9). The current understanding of the group 1 CD1 TCR repertoire emphasizes TCR sequence diversity. T cell clones that recognize CD1a, CD1b, and CD1c express V, D, and J genes that are different from one another (10–14). Lacking TCR sequence motifs, there is currently no basis for organizing group 1 CD1-reactive T cells based on TCR structure. This situation stands in sharp contrast to CD1d, which is also known as the group 2 CD1 system. When CD1d-reactive TCRs from unrelated donors are compared, they show shared sequence motifs that derive from use of a limited range of TCR V and J genes with few nontemplated (N) nucleotides. TCR conservation is a hallmark of NKT cells and is used to define widely recognized T cell subtypes of the CD1d-reactive T cell repertoire.

Type I NKT cells, also known as invariant NKT cells, have a conserved TCR α-chain that uses TRAV10 (also known as Vx24) whereas type II NKT cells also show discernable conservation but do not strictly adhere to sequence motifs.

The apparently differing patterns of diverse or conserved TCRs among group 1 and group 2 CD1 systems, respectively, has been viewed as a fundamental difference between these systems. Based on comparisons to diverse TCRs that recognize MHC proteins, diverse TCRs in the group 1 CD1 system might imply acquired immune function, whereas conserved TCRs on NKT cells formed the basis of early arguments for their innate function (15). Also, in contrast with NKT cells, which are routinely tracked in vivo by staining the defining TCRs with tetrarmers or mAbs (16), there are no widely used equivalent surface staining reagents for group 1 CD1-reactive T cells.

The view that the group 1 CD1-specific TCR repertoire is diverse is currently based on a small number of clones that were generated in different laboratories in response to differing Ags. However, the recent validation of CD1b tetramers provides a method for the rapid generation of clones recognizing the same Ag, derived from genetically unrelated donors under similar conditions (17). Also, Ag-loaded CD1b tetramers allows direct analysis of patterns of TCRs present on polyclonal T cells, which largely bypasses biases that might be caused by technical factors related to the generation of T cell clones in vitro.

Recently, CD1b tetramers bound to the mycobacterial lipid glucose-6-O-monomycocitate (GMM) were used to provide the first example of a conserved TCR pattern in the group 1 CD1 repertoire.
(18). These cells were designed germline-encoded myc4512-specific (GEM) T cells because their TCRs derive from germline sequences encoded by TRAV1-2 and TRAJ9, joined with few N nucleotide additions to create nearly invariant TCR α-chains. Based on this finding, we considered whether the apparent TCR diversity among clones studied to date derives from donor to donor differences in TCR repertoire or instead derives from differences in the Ags and methods used to derive clones. Using tetratmers to systematically analyze T cells from different donors that recognize the same GMM Ag, we identified a previously unknown pattern of TCR conservation that can be detected in vitro and ex vivo. Thus, one CD1b–Ag complex gives rise to at least two consistently observed TCR patterns in humans, providing a basis for using TCR motifs to organize the human CD1b TCR repertoire into at least two compartments that differ with regard to the variable regions used and their affinity for CD1b–glycolipid.

Materials and Methods

Flow cytometry

GMM was purified from Rhodococcus equi as described (19). CD1b monomers (National Institutes of Health Tetramer Facility) were loaded with GMM and assembled into tetratmers (17). Tetratmers were incubated for 30 min at room temperature prior to adding mAbs for an additional 30 min on ice. Flow cytometry data were pregated for lymphocytes based on forward and side scatter. Anti-CD3 and anti-CD4 were from BD Biosciences, the IFN-γ ELISPOT Ab pair was from Mabtech, anti-CD8α was produced in house, anti-CD8β and anti–TRBV4-1 were from Beckman Coulter, and anti–TRA-V1-2 was from BioLegend. Cells were analyzed on a BD FACSCanto II or sorted on an 11-color FACSAria (Becton Dickinson).

T cell cloning and T cell assays

Blood was donated at Massachusetts General Hospital blood bank or from asymptomatic tuberculin-positive subjects with no clinical or radiographic evidence of active tuberculosis (Supplemental Table I), as approved by the Institutional Review Boards of the Lenmuel Shattuck Hospital and Partners Healthcare. Sorted GMM-loaded tetratmer+ T cells were stored overnight in medium containing 0.2 ng/ml IL-15 and plated the next day at 1 cell/well in round-bottom 96-well plates containing 2 × 10^5 irradiated allogeneic PBMCs, 4 × 10^5 irradiated EBV-transformed B cells, 30 ng/ml anti-CD3 Ab OKT3, and 1 ng/ml IL-2, which was added on day 2 of the culture. After 3 wk, the wells with visible growth were restimulated. Clones were tested for binding of GMM-loaded tetratmer by flow cytometry and for Ag specificity in an ELISPOT assay. For ELISPOT assays, cocultures of APCs and T cells were incubated for 16 h in a MultiScreen-IP filter plate (96 wells; Millipore) coated according to the manufacturer’s instructions (Mabtech).

PCR and molecular cloning

RNA was isolated from T cell clones with an RNaseasy kit (Qiagen), and cDNA was synthesized with a QuantiTect reverse transcription kit (Qiagen), including a genomic DNA removal step. V segment usage was determined by PCR using primer set IPS000029 and IPS000030 as described in the ImmunoGencTics web site (http://www.imgt.org) in combination with TRCReC region reverse primer 5′-GTTGATGAGCCTTACCCCTTTG-3′ and TRCBF region reverse primer 5′-GGTGACGGACGACCCCTG-3′. Taq polymerase was used in the supplied buffer (Denville Scientific) under the following cycling conditions: an initial denaturation of 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C, followed by a final elongation step of 7 min at 72°C.

**SKW-3 cell lines**

The full-length TRCReC and TRCBF-chains of CD1b- and MR1-specific T cells were cloned into a 2A peptide-linked pMIG vector, which also contains a gene encoding GFP (3). Vectors were used to transduce the TCR human T cell leukemia line SKW-3. Cell lines were cotransduced with a pMIG vector containing the CD3 subunits ε, ζ, and 6 and γ and harboring 2A peptides (gifted by Richard Berry, Monash University). SKW-3.TCR cells (5 × 10^6) were stained in 50 μl PBS/2% FCS for 30 min at 4°C with 7-aminoactinomycin D viability dye (Sigma-Aldrich), CD3–Pacific Blue (UCHT1; BD Biosciences), and PE-conjugated tetratmers of GMM-loaded CD1b. Cells were washed twice and analyzed on a BD LSIFortessa.

**TCR affinity measurements**

Soluble LDN5 and clone 2 TCR proteins were expressed and purified using methodology described previously (20) and passed in increasing concentrations over GMM-loaded or mock-loaded CD1b coupled to research-grade streptavidin-coated chips in a Biacore 3000. BIACore version 3.1 software (Biacore) was used to fit the data to the 1:1 Langmuir binding model, and the equilibrium data were analyzed with the Prism program for biostatistics, curve fitting, and scientific graphing (GraphPad Software). Affinities of GEM TCRs were published previously (18).

**Results**

**TCR α-chain conservation among tetratmer” clones**

We used CD1b tetratmers loaded with GMM to sort T cells from patients with chronic tuberculosis infection (A22, C58, A14, C39) in an attempt to identify conserved sequence motifs among the tetratmer” clones. Tetratmer” clones (clones 2, 26, 30, 71, 34, 58, 81, and 83) did not express GEM-defining TCR V and J segments seen in tetratmer” clones (Fig. 1B). Furthermore, in contrast to the short N nucleotide regions (0–3 nucleotides) generally found in GEM TCR α-chains (18), we noted long and diverse N nucleotide regions involving 4–10 nucleotides in all tetratmer” clones. Whereas GEM T cell clones have only been detected in the CD4+CD8– pool, tetratmer” clones showed all three patterns of coreceptor expression that are commonly found among mature T cells: CD4+CD8β+, CD4–CD8β+ and CD4–CD8β– (Fig. 1C).

Despite the lack of precise CDR3 α sequence conservation of the type seen in NKT cells and GEM T cells, certain features of TCR α conservation were apparent. All TCR sequences showed the same CDR3 α length, and bias of TRAV genes was observed (Fig. 1B). Among the first eight clones studied, five used TRAV17, which is expressed by ~6.5% of human T cells, based on our survey of published sequences (21). The odds of detecting five or more TRAV17-expressing clones among eight randomly picked ones are 0.10%. Separately, TRAV17 is also expressed by the CD1b–GMM-reactive clone LDN5, which was derived from a leprosy patient many years prior to developing the tetramer methods described in the present study (2). Thus, CD1b–GMM-specific TCRs from clones demonstrated conserved CDR3 α length and TRAV17 usage among many donors studied, but stringent sequence motifs comparable to those in GEM T cells, NKT cells, or mucosal-associated invariant T (MAIT) cells were not observed.

**CD1b conservation**

Despite variation in CDR3 β length and sequence, all but one of the tetratmer” clones from tuberculosis patients expressed TRBV4-1, the same V gene expressed by LDN5 (Fig. 1B). Thus, contrary to the general view that TCRs that recognize group 1 CD1 proteins express diverse αβ TCRs (10–14), sequences of LDN5 and CD1b tetratmer” clones provide evidence for V segment bias in both TCR chains.
The obtained TCR nucleotide and amino acid sequences from unrelated donors emphasize three distinct aspects of the nature of sequence conservation among these TCRs (Fig. 1B). First, we detected three instances of identical α- and β-chain nucleotide sequences present in two clones from the same donor (clones 30 and 71, clones 34 and 58, and clones 81 and 83). The clones were derived from different blood draws or culture wells. Because independent identical rearrangement events in both chains are unlikely, this pattern suggests clonal T cell expansion in vivo prior to the collection of the blood sample. Second, in donor C58, the GMM-reactive T cell clones included two different β-chain sequences that contained TRBV4-1 (Fig. 1B). This pattern is consistent with expansion of similar but not identical T cells that recognize the same Ag within one donor (intradonor conservation). Third, the pattern of GMM-reactive T cell clones expressing a TRAV17-containing α-chain paired with a TRBV4-1–containing β-chain is seen in multiple donors (CC58, A14, C39, and leprosy patient), demonstrating conservation in the TCR α- and β-chain V gene expression among unrelated donors (interdonor conservation). LDN5 was previously thought to be a unique type of T cell derived from the skin of one leprosy patient, but these data show that GMM-reactive T cells from other patients with mycobacterial infection express similar TCRs. Based on patterns of intradonor and interdonor conservation of TCRs that resemble the TCR expressed by LDN5, we designated GMM-specific, tetramerint, TRAV4-1+ cells as LDN5-like T cells.

LDN5-like T cell populations ex vivo

To determine whether LDN5-like T cells were detectable ex vivo by functional responses as polyclonal populations, we tested freshly isolated T cells from a tuberculosis patient and healthy donors recruited in a blood bank (BB). Experiments addressed the potential shared use of TRBV4-1, which was seen in LDN5 and all but one tetramerint clone (Fig. 1B). Using an mAb that specifically

**FIGURE 1.** LDN5-like T cell clones show TCR conservation. (A) CD1b tetramers were mock treated or GMM treated prior to staining of T cell clone 83 and GEM T cell clone 1. (B) Nucleotide sequences (lowercase) and amino acid sequences (uppercase) of CDR3 regions of the TCRs of tetramerint GEM clone 1 (17), LDN5 (9), and tetramerint clones (clones 2, 26, 54, 71, and 83). Light gray, V segment–derived nucleotides; dark gray, J segment–derived nucleotides; boxed areas, non-germline–encoded nucleotides in the α-chain. (C) Staining with Abs against CD4, CD8α, and CD8β shows that tetramerint clones can be CD4+, CD8αβ+, or CD8α+CD8β−. For (A), two experiments were performed for both clones that are shown. Tetramer staining of clone 83 is representative of six comparable clones, and staining of clone 1 is representative of three comparable clones. For (C), each staining was performed once.
recognizes TCR β-chains with TRBV4-1, sorted CD3+TRBV4-1+ T cells were compared with CD3+TRBV4-1- cells. Because CD3+TRBV4-1- cells might include GEM T cells, which express TRAV1-2, we excluded TRAV1-2+ cells, expecting CD1b–GMM-reactive cells to be in the CD3+TRBV4-1+ gate only. CD3+ TRBV4-1+ cells from a tuberculosis patient (C32) and a BB donor (BB8) were activated by GMM in a CD1b-dependent manner to produce IFN-γ (Fig. 2). No response was detected using equal numbers of CD3+TRBV4-1- TRAV1-2+ T cells. T cells from a second BB donor (BB40) failed to recognize GMM, indicating

FIGURE 2. TRBV4-1–expressing T cells are enriched for CD1b–GMM–specific T cells. T cells of a latent tuberculosis patient (C32) or random BB donors (BB8, BB40) were sorted based on expression of CD3 (gate not shown), TRAV1-2, and TRBV4-1 (left panels). An IFN-γ ELISPOT assay was performed using equal numbers of the indicated sorted T cells that were stimulated directly ex vivo with K562 cells that were transfected with CD1b or CD1c in the presence or absence of GMM. The experiment was performed twice on donor C32 and once on donors BB40 and BB8.

FIGURE 3. Cytokine production by T cell clones. Luminex assay of cytokines in supernatants of LDN5-like clones (LDN5, clones 2, 26, and 34), GEM T cell clones (clones 1 and 42), control clones (clones 50 and 101), and CD1a-specific T cell clones that recognize didehydroxymycobactin (clones 5, 15, and 32). T cells were left unstimulated or were stimulated for 16 h with the phorbol ester PMA and ionomycin. Data are from one Luminex experiment using pooled supernatants from triplicate wells. Analytes that reached levels that were above the limit for reliable detection are indicated with an asterisk.
that GMM-specific cells, if present, were below the threshold of detection. TRBV4-1 is used by many other T cells, so sorting TRBV4-1+ T cells is expected merely to enrich for LDN5-like T cells, a conclusion that is supported by the rate of T cell response (Fig. 2). Nevertheless, CD1b–GMM-reactive T cells are detectable ex vivo without the use of tetramers, and they are enriched among TRBV4-1+ T cells (Fig. 1).

CD1b-specific cells express variable cytokine profiles

We next sought to determine whether LDN5-like T cells and GEM T cells express any characteristic cytokine profile. Supernatants of T cell clones stimulated with PMA and ionomycin were analyzed in a Luminex multiplex cytokine assay (Fig. 3). Clones were organized into groups that met criteria for LDN5-like T cells (LDN5, clones 2 and 34), GEM T cells (clones 1 and 42), and clones

Table I. TCRs define two subsets in the CD1b-reactive repertoire

<table>
<thead>
<tr>
<th>Defining Criteria</th>
<th>GEM</th>
<th>LDN5-like</th>
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<tbody>
<tr>
<td>TCR α-chain</td>
<td>TRAV1-2, TRAJ9</td>
<td>TRAV17</td>
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<tr>
<td>Stringency of motif</td>
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<td>Bias, uniform length</td>
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<td>TCR β-chain</td>
<td>TRBV6-2</td>
<td>TRBV4-1</td>
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<td>Bias, variable length</td>
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<td>Coreceptors</td>
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<td>CD4, CD8, or DN</td>
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<tr>
<td>Affinity</td>
<td>High</td>
<td>Intermediate</td>
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recognizing CD1a and the mycobacterial lipopeptide dideoxymycobactin (clones 5, 15, and 32). As controls, we tested clones with irrelevant Ag specificity (clones 50 and 101) or unstimulated LDN5 T cells. The assay included cytokines characteristic for the helper T cell subsets Th1, Th17, and Th2, the acute phase and antiviral cytokines IL-6, TNF, LT-α, IFN-α, and IFN-β, the chemokines IP10 (CXCL10), XCL-1, MPI1β (CCL4), I-309 (CCL1), and TARC (CCL17), and the negatively regulating mediators IL-10 and the IL-1 receptor antagonist. The data show that all stimulated clones expressed TNF and IFN-γ, a pattern previously observed for GEM T cells that is consistent with a proposed antimicrobial effector function of GMM-specific T cells. LDN5-like T cell clones did not show shared patterns that define them as Th2- or Th17-like T cells, but instead show somewhat varied cytokine profiles, which suggests that LDN5-like T cells may exist as functionally specialized subsets.

TCR avidity for CD1b–glycolipid

A consistently observed difference between LDN5-like and GEM T cells is that the former show intermediate staining with CD1b–GMM tetramer, and the latter stain brightly (Fig. 1A). Factors that contribute to the tetramer binding by a T cell clone are the density of TCR expression at the cell surface, the affinity of the TCR for CD1b, and contributions by receptors other than the TCR. Therefore, we sought to measure the avidity and affinity of TCRs using quantitative or direct methods. To control TCR expression level, we transduced SWK-3 cells with GEM TCRs (clones 1, 21, and 42) or LDN5-like TCRs (LND5, clones 2 and 34) and measured tetramer staining within narrow CD3 windows over a broad range of added tetramer, as shown in detail for clone 21 (Fig. 4A, upper panels). In all cases and across broad ranges of tetramer concentration, SWK-3 cells with GEM TCRs stained more intensely with CD1b–GMM tetramer than did cells expressing equivalent density of LDN5-like TCRs (Fig. 4A, lower panel, 4B).

As a complementary approach with additional negative controls, SWK-3 cells were transduced with GFP-tagged MAIT TCR (M33.20), CD1b-mycoid–reactive TCR (clone 18), LDN5-like TCRs, and GEM TCRs. Cells were gated for equivalent GFP and CD3 staining and validated for CD1b–GMM tetramer binding (Fig. 4B). The amount of tetramer used in this experiment was 750 ng, which was nonsaturating (Fig. 4A). Again, SWK-3 cells expressing TCRs of GEM T cell clones 1, 21, and 42 showed high tetramer staining, whereas LDN5-like TCRs mediated intermediate tetramer staining. These data control for TCR expression, cell surface effects, and tetramer loading with Ag, providing quantitative measurements in support of the conclusion that LDN5-like TCRs show lower avidity for CD1b–GMM compared with GEM TCRs in all cases studied.

TCR affinity for CD1b–glycolipid

For direct measurement of TCR affinity to CD1b–glycolipid, we generated the disulfide-linked, transmembrane region–truncated recombinant TCRs of LDN5 and the LDN5-like clone 2 (20). The proteins were of the expected apparent m.w. under reducing and nonreducing conditions (Fig. 4C). Soluble TCR was passed over immobilized CD1b to determine the affinity values. Both the LDN5 TCR and the clone 2 TCR bound to GMM-treated, but not mock-treated, CD1b. We determined a Keq of 39.4 ± 8.0 μM and 19.4 ± 0.88 μM for LDN5 and clone 2, respectively (Fig. 4D). These Keq values are comparable to what are considered intermediate affinity conventional peptide-specific TCRs (22), and they are 20- to 40-fold higher than previously reported values for GEM TCRs made using the same method (Fig. -E) (18). Thus, compared with GEM T cells, LDN5-like T cells consistently exhibit lower affinity and avidity binding to GMM-loaded CD1b complexes. These data link the less stringent TCR motifs with lower affinity Ag recognition in a pattern that is reminiscent of type II NKT cells (23, 24).

Discussion

Collectively, these results identify LDN5-like T cells as cells expressing diverse coreceptors and TRAV17- and TRBV4-1–biased TCRs, which mediate intermediate affinity interactions with CD1b and GMM. Accordingly, LDN5, a T cell clone that was previously considered a single TCR within the diverse T cell repertoire that recognizes group 1 CD1, now appears to be a “generalizable anecdote” that identifies a previously unknown pattern of interdonor TCR conservation. These observations run counter to widespread views that the group 1 CD1 TCR repertoire is so complex that TCRs cannot be used to meaningfully organize the repertoire into subsets. Instead, systematic analysis of T cell clones recognizing one CD1b–Ag complex revealed that the CD1b repertoire consists of at least two definable compartments, GEM T cells and LDN5-like T cells. Sequence conservation among LDN5-like TCRs relays predominantly on the TCR β-chain, whereas the lower affinity and α-chain conservation is lower than the nearly identical TCR α-chains present in GEM TCRs. Also, whereas all known examples of GEM T cells are CD4+CD8−, LDN5-like T cells seem to have more diverse coreceptor expression with examples of CD4 and CD8 single-positive, as well as double-negative, T cell clones present in the panel (Fig. 1C, Table I).

Thus, when experimental variables related to differing Ags and cloning methods are eliminated, the first two efforts using tetramers to systematically characterize CD1b-reactive TCRs revealed two clear patterns of interdonor conservation. Two different types of TCRs recognize the same GMM Ag, high-affinity TCRs that use TRAV1-2 and TRAJ9, and intermediate affinity TCRs that use TRAV17 and TRBV4-1. Thus, TCR diversity is not an intrinsic and universal characteristic of group 1 CD1-reactive T cells. The high-affinity and highly stringent sequence motifs of GEM T cells compare with type I NKT cells, whereas the lower affinity and motif stringency in LDN5-like T cells compare to type II NKT cells. Indeed, questions now arise as to whether the interdonor conservation is typical of the repertoire of T cells recognizing the nonpolyomorphc Ag-presenting molecule, CD1b. To date, GMM is the only CD1b Ag studied systematically, so tetramer-based analysis of other known lipid ligands of CD1b, including phosphatidylinositol monoside, sulfoglycolipid, free mycolate, glycerol mycolate, or phosphatidylinositol provide a means to test this new model. If interdonor conservation exists broadly, it might provide a practical means to diagnose infection or other diseases that involve lipid Ags, and it has implications for where CD1b-reactive T cells fall in the spectrum of innate and acquired immunity.

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Disclosures

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References


