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Human autoreactive T cells recognize CD1b and phospholipids

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Abstract

In contrast with the common detection of T cells that recognize MHC, CD1a, CD1c or CD1d proteins, CD1b autoreactive T cells have been difficult to isolate in humans. Here we report the development of polyvalent complexes of CD1b proteins and carbohydrate backbones (dextramers) and their use to identify CD1b autoreactive T cells from human donors. Activation is mediated by $\alpha\beta$ T cell receptors binding to CD1b-phospholipid complexes, which is sufficient to activate autoreactive responses to CD1b-expressing cells. Using mass spectrometry and T cell responses to scan through the major classes of phospholipids, we identified phosphatidylglycerol (PG) as the immunodominant lipid antigen. T cells did not discriminate the chemical differences that distinguish mammalian PG from bacterial PG. Whereas most models of T cell recognition emphasize TCR discrimination of differing self and foreign structures, CD1b autoreactive T cells recognize lipids with dual self and foreign origin. PG is rare in the cellular membranes that carry CD1b proteins. However, bacteria and mitochondria are rich in PG, so these data point to a more general mechanism of immune detection of infection- or stress-associated lipids.

Significance statement

Peptides are known to be targets of autoreactive T cells that can cause autoimmune diseases. Here we show that human T cells recognize self lipids displayed on the surface of cells by CD1b proteins. A particular lipid that is recognized by CD1b autoreactive cells, phosphatidylglycerol, is present in mammalian mitochondria and in bacteria. Therefore, these data define a phospholipid autoantigen that is released during mitochondrial stress and bacterial infection.

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Introduction

Whereas most studies of the human $\alpha\beta$ T cell response have focused on peptide antigens bound to MHC class I or II molecules, human CD1 proteins represent a parallel system of antigen display that allows T cells to recognize and respond to lipids. Like MHC II proteins, human CD1 antigen presenting molecules (CD1a, CD1b, CD1c, CD1d) are expressed at high density on the surface of professional antigen presenting cells (APCs), such as myeloid dendritic cells (DCs), Langerhans cells and B cells (1). CD1 proteins can capture self lipids in the endoplasmic reticulum or the secretory pathway, and the cytoplasmic tails of CD1b, CD1c and CD1d proteins mediate transport to lysosomes for antigen capture at low pH (2). However, unlike MHC proteins, the inner surface of CD1 clefts are lined by hydrophobic, rather than polar or charged amino acids, so that there is almost no overlap in the chemical nature of antigens displayed by MHC and CD1 proteins. Thus, the spectrum of natural antigens for T cells is broader than previously thought, creating a situation in which lipids might be developed as the basis for detecting or activating human T cells.

In addition to CD1d, humans and most other mammals express CD1a, CD1b and CD1c proteins, which are known as group 1 CD1 proteins. Prior work using human T cell clones (3-7) and the recently developed human CD1a, CD1b and CD1c tetramers (8-10) have demonstrated the existence of mycobacteria-reactive T cells, including polyclonal T cells with stereotyped TCRs, known as germline-encoded mycolyl lipid-reactive (GEM) T cells (11) and LDN5-like T cells (12). Currently, nearly all foreign antigens presented by CD1b, including mycolic acid, glucose monomycolate, glycerol monomycolate and sulfoglycolipids, are selectively synthesized by *Mycobacterium tuberculosis* and related mycobacterial species. Whether or not CD1b

functions more broadly in human T cell responses to other types of bacteria is unknown. To address the breadth of pathogens recognized by CD1b-mediated T cells, we studied human T cell responses to pathogenic *Salmonella*, *Staphylococcus* and *Brucella* species. CD1b tetramers have recently been proven to markedly enhance the rate of detection and recovery of CD1b-reactive T cells (8, 11, 12). However, tetramers are not usually used for antigen discovery because they require that homogenous antigen preparations be loaded onto MHC or CD1 proteins. That is, two or more arms of the tetramer must be loaded with the same or similar antigen, which is usually needed to create a multimeric ligand with sufficient avidity to bind TCRs.

However, we reasoned that bacterial lipid mixtures could be screened for T cell response using higher order CD1b multimers formed on dextran polymer backbones, which are known as dextramers. This strategy is based on the premise that the higher valence can increase the chance that two or more of the CD1b proteins capture equivalent lipids to create TCR binding epitopes. Whereas mycobacteria express at least 119 classes of lipids defined by Lipid Maps and the MycoMass dataset (13), *Staphylococcus*, *Salmonella* and *Brucella* species produce a much simpler lipid envelope whose content is dominated by membrane phospholipids, which are polar and elute in methanol from normal phase silica columns. Further, prior efforts to discover lipid antigens for CD1 proteins have isolated antigens from methanol eluents of silica columns (4, 14). Therefore, use of methanol eluents from bacteria represented a semi-targeted approach that seems to enrich for amphipathic molecules that possess CD1 binding properties. This screening approach succeeded in reproducibly detecting human $\alpha\beta$ T cells responding to all three bacterial species, thereby expanding the scope of pathogens recognized by the CD1b system. Surprisingly, studies aimed at solving the chemical structure of the lipid antigens showed that the optimal molecular targets were phospholipids that are synthesized both in mammalian cells and

pathogens. Further, recognition of self lipids by T cells was accompanied by autoreactivity to CD1b expressed on human cells. We show that the molecular basis of self and foreign lipid antigen recognition required $\alpha\beta$ TCR binding to CD1b-phospholipid complexes, but data pointed away from differences in self and foreign lipid structure as the determinant of T cell response. Instead, these studies showed that CD1b-presented antigens are rare in the membranes from which CD1b proteins capture self lipids, but are highly abundant in bacteria.

Results and Discussion

To isolate T cells that recognize microbial antigens derived from pathogens other than mycobacteria, we treated CD1b proteins with lipid extracts from *Staphylococcus aureus*, *Brucella melitensis*, and *Salmonella Typhimurium*. CD1b-lipid complexes were conjugated to dextran polymer backbones (dextramers) with an approximate valence of 10. Mock-treated or bacterial lipid-treated dextramers were used to stain PBMC isolated from healthy blood bank donors. Microbial lipid-treated CD1b dextramer⁺ T cells could be detected in the blood at low frequency, comparable to that of naive, peptide-specific T cells or glucose monomycolate-specific T cells (~1 in 10⁵). After flow cytometric sorting and expansion of cells in culture, polyclonal T cell lines from two donors were derived that stained with bacterial lipid-loaded CD1b dextramers (**Figure 1a**). CD1b dextramer staining was bright, and no staining was seen using CD1a dextramers as a control. Using this method, T cell lines could be generated in response to lipids from all three species of bacteria and in each of two donors (A25, BC8) (**Figure 1b**). In functional assays of T cell response, IFN- γ ELISPOT showed that *S. aureus*, *B. melitensis*, and *S. Typhimurium* lipids stimulated cytokine responses when APCs (K562 cells) were transfected to express CD1b but not in response to K562 cells expressing CD1a (**Figure**

2a). Thus, CD1b dextramers represent a new and reliable method to detect human T cells that respond to CD1b, demonstrating clear reactivity to lipids from three non-mycobacterial species.

Surprisingly, all four T cell lines also showed clear responses to CD1b-transfected K562 cells in the absence of bacterial extract, although bacterial lipids did increase IFN- γ production in some cases (**Figure 2a**). This finding was unexpected because the T cell lines were derived using bacterial lipids. More generally, the finding was notable because studies over the past 20 years have produced little evidence for T cell autoreactivity to CD1b proteins. CD1b-mediated T cell responses to defined antigens can be generated through in vitro immunization with self or foreign antigens (3, 7, 15), and a mouse TCR recognizes human CD1b (16). However, CD1b-mediated human T cell autoreactivity to endogenously expressed lipids in human cells is limited to a few clones (17, 18), in contrast to the large number of known MHC-, CD1a-, CD1c- and CD1d- autoreactive clones. Further, in systematic analyses of CD1 autoreactivity using large panels of T cell clones (19, 20) or in polyclonal T cells (20), T cell autoreactivity to CD1b was very low or undetectable under conditions in which CD1a, CD1c and CD1d autoreactivity are prominent. We confirmed the lack of autoreactivity of another CD1b-glucose monomycolate (GMM) specific cell line, known as LDN5, under conditions in which the four newly derived T cell lines showed strong autoreactivity. Conversely, we demonstrate the lack of recognition of GMM by these autoreactive cell lines (**Figure S1**). Together, these experiments demonstrated that autoreactivity and CD1b-GMM specificity are non-overlapping T cell specificities and that CD1b autoreactivity is not a general feature of T cells that recognize foreign antigens presented by CD1b.

The T cell lines were named after the donor and antigen source, and in three of the four lines (BC8Staphylococcus, A25Salmonella, A25Staphylococcus), autoreactivity to CD1b could

be confirmed using a second APC type, the B cell lymphoblastoid cell line C1R, transfected with CD1b (C1R.CD1b). In a multiplex cytokine array of 38 effector molecules, CD1b-dependent secretion of IL-2, IL-4, IL-8, IL-13, TNF, IFN- γ , MIP-1 α , MIP-1 β , oncostatin M (OSM), CXCL13 (B lymphocyte chemoattractant, BLC), GM-CSF, sTNF-RII was demonstrated (**Figure 2b**). However, upon stimulation with C1R.CD1b, the line BC8Staphylococcus did not produce cytokines in concentrations that were more than twice higher than seen with mock-transfected C1R cells. Bacterial lipid mixtures might activate T cells via many receptors, but we observed TCR downregulation and CD25 upregulation on nearly all cells, which is characteristic of TCR-mediated signals (**Figure 2c**). Whereas these mechanistic experiments were performed with two types of CD1b transfectants, we also observed a strong response to monocyte derived DCs, which meaningfully model the function of natural CD1b-expressing APCs (**Figure S2 and S3**). Collectively, these data provide clear evidence for the existence of human T cells that are autoreactive to CD1b proteins.

Next we sought to understand why T cells isolated using lipids from three differing bacterial species could also recognize CD1b proteins expressed on cells that were not exposed to bacterial lipids. This might be explained if TCRs directly bind CD1b without regard to the loaded lipid, similar to a recently identified mechanism by which a CD1a autoreactive TCR binds an unliganded facet of the CD1a surface (21). However, lipid-independent recognition was unlikely since bacterial lipids strongly augmented dextramer staining of all four T cell lines (**Figure 1b**). Interestingly, one subtle aspect of dextramer staining patterns hinted that endogenous self lipid antigens can also mediate TCR binding to CD1b. Two T cell lines (BC8Staphylococcus, BC8Brucella) showed higher staining for mock-loaded CD1b dextramers as compared with CD1a dextramers (**Figure 1b**) while two other T cell lines stained with nearly

identical intensity with mock and bacterial lipid treated CD1b dextramers. This pattern could be explained if mock-loaded tetramers produced in mammalian cells carry lipid autoantigens. Therefore, a potentially important implication of these results is the possible existence of endogenous self lipid antigens for the CD1b system that are recognized in a cross-reactive manner by T cells that can also detect bacterial lipids.

To test this hypothesis, highly purified or synthetic phospholipids, including phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were used to probe their ability to mediate CD1b dextramer binding to T cells. Although it is less abundant in mammalian cells, we also tested lysophosphatidic acid (LPA) because it is an antigen or permissive ligand in the CD1d and CD1a systems. Lines A25Salmonella and BC8Staphylococcus generated distinct patterns of T cell response to structurally related phospholipids (**Figure 3a**). Both lines showed reactivity towards PA, but not LPA, showing that two fatty acids are required for recognition. The two lines showed markedly differing staining with PI and PC but both stained with multiple lipids and showed very strong staining with PG. Mammalian cells and bacteria both produce PA and PG, so these lipids can be considered antigens with dual self and foreign origin, which might explain the mixed patterns of recognition of untreated and bacterial lipid-treated dextramers (**Figure 1b**) or CD1b proteins expressed on mammalian cells (**Figure 2a**). Further, because many genera of bacteria other than the three studied here overexpress PG as compared to mammalian cells, this lipid might represent a general target for bacterial recognition by T cells.

To more directly test this hypothesis, methanol eluates from *S. aureus* and *S. Typhimurium* were subjected to nanoelectrospray ionization mass spectrometry in the negative mode. We detected strong signals corresponding to the expected mass of the [M-H]⁻ ion of PG in

S. aureus (m/z 714.4) and *S. Typhimurium* (m/z 733.4), with detailed structures confirmed by collision-induced dissociation mass spectrometry (CID-MS) (**Figure 3b, Supplemental Figure S4**). The dominant form of PG that was detected in *S. Typhimurium* contains a C17:1 and a C16 acyl chain, and the dominant form of PG in *S. aureus* contains a C17 and a C15 acyl chain, which is consistent with published data (22). Thus, PG is present in the methanol eluates loaded onto CD1b dextramers, and it is notable that ions corresponding to PG were the dominant phospholipid identified in these lipid preparations (**Figure 3b**). This finding is consistent with prior reports that PG is abundant in many bacteria, and that it is a quantitatively dominant phospholipid in *S. aureus* (23) and *S. Typhimurium* (24).

To potentially explain the higher dextramer binding (**Figure 1**) and T cell activation (**Figure 2**) upon treatment with bacterial lipids, we considered whether subtle differences in PG structure could control reactivity. In fact, there are known, systematic differences in fatty acid content, position (*sn1* or *sn2*) and chirality (*R* or *S*) of the glycerol head group in mammalian and bacterial PGs. In particular, fatty acyl units of bacterial but not mammalian PGs have methyl branching or cyclopropyl groups, and such substitutions have been shown to promote recognition of lipids in some (6, 25-27) but not in all (28) situations. CD1b dextramers loaded with either pure synthetic PGs matching the common mammalian form or stereoisomers present in *Corynebacterium glutamicum*, or natural PG from *Listeria monocytogenes* or *Escherichia coli* all showed comparable dextramer staining intensity, which largely ruled out this fine specificity hypothesis (**Figure 3c-d**).

However, a second possible basis for self-foreign discrimination is the known difference between PG concentrations in mammalian and bacterial cells. PG is relatively scarce in mammalian cells, comprising between 1 and 5% of the total phospholipid content in mammalian

tissues or cultured cells (29). Further, unlike other mammalian phospholipids, PG is synthesized in mitochondria, where it is used in cardiolipin biosynthesis and maintains an organelle-specific distribution pattern (30, 31). PG cannot be detected in the membranes that comprise CD1b⁺ subcellular compartments, including the endoplasmic reticulum and Golgi apparatus, and is present in trace amounts in the plasma membrane (32). In contrast, PG is highly abundant in bacterial membranes. For example, in *S. Typhimurium*, *E. coli*, *S. aureus* and *B. subtilis* it accounts for 18, 19, 58, and 70% of the total phospholipids respectively (23, 24, 33). Thus, PG can be considered a dual self and foreign molecule, but its presence at high levels in bacterial membranes represents a plausible means of detection as a foreign antigen, representing a general signature of microbial infection.

Line A25Salmonella showed a pattern of staining in which each of several phospholipids mediated dextramer binding in a defined hierarchy (**Figure 3a**). This pattern might have been caused by true cross-reactivity of one TCR with several phospholipids, or instead by multiple TCRs within an oligoclonal T cell line, each of which specifically recognizes one phospholipid type. To distinguish these possibilities and formally identify the receptor mediating response, we cloned two TCRs from A25Salmonella, which were named PG10 and PG90 based on the recognition of PG by the T cell line they were derived from. The PG10 TCR was comprised of TRAV13-1, TRAJ8, TRBV4-1, and TRBJ2-3, and PG90 expressed TRAV26-1, TRAJ13, TRBV7-8, and TRBJ2-2 TCR gene segments (**Figure 4a**). The TCRs were retrovirally transduced into Jurkat clone 76 cells that lack endogenous TCR α and β chains (34). Jurkat.PG10 and Jurkat.PG90 cells recognized C1R cells transduced with CD1b, but not wild type C1R cells, formally establishing that these two TCRs mediate CD1b autoreactivity (**Figure 4a**).

To determine antigen specificity of these clonal TCRs, we carried out staining experiments with CD1b tetramers. Whereas dextramers outperformed tetramers when loaded with mixed lipids, tetramers were adequate when loaded with pure lipid ligands (**Figure 4**). Tetramers were mock loaded and thus contain a mixture of endogenous self phospholipids, or were loaded with defined glycerophospholipids, or were loaded with known CD1b ligands that are not glycerophospholipids (sphingomyelin, ganglioside T1b, sulfatide, and glucose monomycolate). The resulting staining patterns supported several key conclusions. First, both Jurkat.PG10 and Jurkat.PG90 stained with more than one CD1b-phospholipid tetramer, demonstrating true antigen cross-reactivity to phospholipids for single TCRs. Second, despite distinct TCR gene usage, the pattern of reactivity observed for PG10 and PG90 was highly similar in both transfectants and the parental A25Salmonella line. The two TCRs have minor differences in antigen specificity: the strongest staining was seen in response to PG, followed by PA or PS, but only PG10 stained with PI-loaded tetramers. Thus, the observed autoreactivity of A25Salmonella is likely mediated by two or more distinct TCRs that bind CD1b-PG complexes, with detectable but lower avidity for CD1b complexes made with other membrane phospholipids.

More generally, we conclude that human $\alpha\beta$ TCRs mediate CD1b autoreactivity and do so through recognition of CD1b bound to self lipids, including PG and PA. The phospholipids PC and PI were known to bind to CD1b, but thought to have a chaperone-like function instead of interacting with TCRs or providing activation signals (35, 36). As the concept of $\alpha\beta$ T cell autoreactivity to CD1b emerges, the particular hierarchies of lipids that mediate CD1b-TCR binding provides some initial clues on the origin of autoantigens as well as testable hypotheses regarding a cellular mechanism of CD1b autoreactivity. Combining results obtained with soluble

CD1b produced by 293T cells and CD1b expressed on K562 or C1R cells, dextramer and tetramer staining (**Figures 1, 3a, 3c, 4**) and T cell activation (**Figures 2, 4**) depend on whether CD1b proteins display a homogeneous or heterogeneous cohort of self lipids and the extent to which these can either promote or block TCR binding to CD1b. Interestingly, mock-loaded CD1b tetramers show detectable staining on both Jurkat.PG10 (MFI=371) and Jurkat.PG90 (MFI=489). Furthermore, the non-glycerophospholipid ligands did not enhance this staining and in some instances caused reduced staining compared to mock-loaded tetramers, raising the possibility that some CD1b bound ligands may be inhibitory (**Figure 4b**). Therefore, similar to models recently proposed for CD1a (20, 21), cellular autoreactivity is plausibly controlled by the ratio of activating to blocking ligands on the surface, rather than the failure to recognize self lipids and the introduction of foreign lipids during infection.

Overall PG is much more abundant in bacteria than in mammalian cells. Therefore, our favored hypothesis is that bacterial infection represents a means for introducing antigens into the host. However, prior studies also show that in mammalian cells, PG is present at low concentrations and is thought to be exclusively synthesized in mitochondria. Thus, PG may normally be largely inaccessible to CD1b in a steady state, but appear in settings that cause mitochondrial stress. Therefore, exposure to PG-rich bacteria or activation of stress pathways that release rare self antigens both become candidate mechanisms of phospholipid-mediated T cell activation.

Materials and Methods

Antigens

Staphylococcus aureus was cultured in Brain-Heart Infusion broth, and *Salmonella* Typhimurium was cultured in Luria Broth. *Brucella melitensis* human isolate, strain X10017283-001 (CVI) was cultured in tryptic soy broth. Lipid extracts were prepared by extracting bacterial pellet with chloroform/methanol 1:2 (V:V) for two hours at room temperature, followed by chloroform/methanol 2:1 (V:V). Fractions were generated that are enriched for neutral lipids, polar lipids, and charged lipids by loading total lipid extracts on disposable silica columns (Alltech, 10 mg) and eluting with chloroform, acetone, and methanol, respectively. C32 glucose monomycolate was purified from *Rhodococcus equi*. *Listeria monocytogenes* C17 C15 PG was purified as described. C18:1 C16 RR PG and C18:1 C16 RS PG, which are commonly found in *Corynebacterium glutamicum*, were synthesized as described (27). The following commercially available lipids were used: PS from brain (Avanti 840032c), C16 C18:1 PC (Avanti 850475c), C16 C18:2 PI (Avanti 840044c), C16 C18:1 PA (Avanti 840857), C18:1 LPA (Avanti 857130c), synthetic C16 C18:1 PE (Avanti 850757c), synthetic PG C18 C18:1, which is commonly found in mammalian cells (Avanti 840503), *E. coli* C16 C17 PG (Avanti 841188p), N-nervonoyl-D-erythro-sphingosylphosphorylcholine (SM) (Avanti), Mono-Sulfo Galactosyl(β) Ceramide (C24:1 Sulfatide) (Avanti), Trisialoganglioside (GT1b) was obtained from Sigma-Aldrich.

Flow cytometry

For the generation of dextramers, CD1a and CD1b monomers produced in the human cell line HEK 293T (NIH tetramer facility) were loaded with antigens and assembled into phycoerythrin- or allophycocyanin-labeled dextramers as described (8, 9). Dextramers were incubated with cells for 30 minutes at room temperature prior to adding monoclonal antibodies for an additional 30

minutes on ice. For tetramers, DNA encoding for the ectodomain of human CD1b fused to a C-terminal BirA and hexa-His tag, or $\beta 2m$, was cloned into the vector pHLsec and expressed by transient transfection of mammalian HEK293S.GnTI cells (37). CD1b was purified using immobilized metal affinity chromatography and gel filtration, and biotinylated with BirA enzyme (Avidity) as per manufacturer's protocol. Lipid-loaded biotinylated monomers were tetramerized by the addition of streptavidin-PE (BD Pharmingen) at a 4:1 molar ratio. For loading, lipid antigens were prepared in 0.5% tyloxapol in Tris-buffered saline pH 8.0 and loaded into CD1b at a 6:1 lipid:protein molar ratio at room temperature overnight. Flow cytometry data were pre-gated for lymphocytes based on forward and side scatter. The following antibodies were used: CD3 ϵ -FITC (SK7, BD biosciences) CD3 ϵ -allophycocyanin (UCHT1, eBioscience and Becton Dickinson), CD69-phycoerythrin, (FN50, Becton Dickinson), and CD25-FITC (BC96, Biolegend). Cells were analysed on a FACS Canto II or LSRFortessa, and sorted on a FACS Aria III (Becton Dickinson). Data were analysed using FlowJo (Treestar Inc.).

T cell lines and T cell assays

Leukoreduction collars from random blood bank donations were obtained from the Brigham and Women's Hospital Specimen Bank. Sorted CD1b dextramer⁺ T cells were stored overnight in medium (10) containing 0.2 ng/ml IL-15 and plated the next day at 1000 cells/well in round bottom 96-well plates containing 2×10^5 irradiated allogeneic peripheral blood mononuclear cells, 4×10^4 irradiated Epstein-Barr virus transformed B cells, 30 ng/ml anti CD3 antibody OKT3 and 1 ng/ml of IL-2 which was added on day 2 of the culture. After 2 weeks, CD1b dextramer⁺ cells were sorted and expanded again. For ELISPOT assays, co-cultures of 2×10^4 APCs and 3×10^3 T cells were incubated for 16 h in a Multiscreen-IP filter plate (96 wells; Millipore) coated according to the manufacturer's instructions (Mabtech). An unpaired two-tailed

t test was used for comparisons of two groups. For Luminex multiplex cytokine assays, 1×10^5 T cells and 1×10^5 CD1b expressing or non-expressing C1R cells were mixed, or alternatively, cells were stimulated with PMA and ionomycin, and supernatants were collected after 24 hours.

TCR cloning and transfer

TCR α and β chains were determined by PCR and Sanger sequencing as described previously (12). An antibody against TRBV4-1 (ZOE, Beckman Coulter) was used to sort a subpopulation of the T cell line A25Salmonella. Cell lines expressing $\alpha\beta$ TCRs were generated as previously described (37). Briefly, Jurkat-76 cells were transduced with pMIGII vector containing full length PG10 or PG90 TCR α and β chains, separated by a 2A-linker. For co-culture assays, Jurkat-76 T cells lines were either cultured with wild type C1R cells or with C1R cells expressing CD1b (3×10^4 of each cell line) for 6–12 h in round-bottom 96-well plates and their activation status measured by flow cytometry to detect CD69 upregulation.

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Author contributions

IVR, JR, DG, DBM designed research; IVR, TVB, TH, TYC, AU, GN performed research; BW, RT, MB, JA, PW contributed new reagents or analytic tools; IVR, TVB, TH, TYC, AU, GN, VC, SH, JR, DG, DBM analyzed data; and IVR, RT, AU, GN, VC, JR, DG, DBM wrote the paper.

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Figure legends

Fig. 1 Generation of T cell lines that recognize bacterial lipids presented by CD1b

(a) T cells from donor A25 that bind to CD1b dextramers loaded with *Salmonella* Typhimurium lipids were enriched by multiple rounds of FACS sorting and expansion until they reached near-uniform binding of bacterial lipid extract-loaded CD1b dextramers. (b) Flow cytometric analysis of the cell line shown in panel a, as well as a *Staphylococcus aureus* lipid-specific cell line from the same donor, using the indicated CD1 dextramers. Two additional T cell lines were analyzed that were specific for *Brucella melitensis* and *Staphylococcus aureus* lipids, derived from donor BC8. For each T cell line, at least five flow cytometric experiments were performed with similar results.

Fig. 2 Bacterial lipid-specific T cells are autoreactive to CD1b proteins on mammalian cells

Bacterial lipid-specific T cell lines were stimulated with K562 cell lines stably transfected with the indicated CD1 isoform (a is CD1a, b is CD1b), with or without exogenously added *S. Typhimurium* (Sal.) or *S. aureus* (Staph.) lipids in an IFN- γ ELISPOT assay (a) or stimulated with mock transfected or CD1b transfected C1R lines in the absence of exogenous antigen, upon which supernatants were harvested after 24 hours and analyzed for cytokine content. (b) In panel b, only those cytokines are shown that were produced upon stimulation with C1R.CD1b but not upon stimulation with mock transfected C1R cells. In addition to the cytokines shown, 11 analytes were produced upon stimulation with PMA and ionomycin, but not upon stimulation with C1R.CD1b: IL-3, IL-6, IL-9, IL-10, IL-17, IL-22, IL-31, LT- α (TNF- β), CCL1 (I-309), CXCL10 (IP-10), latency-associated peptide (LAP) of TGF- β . Production of the following

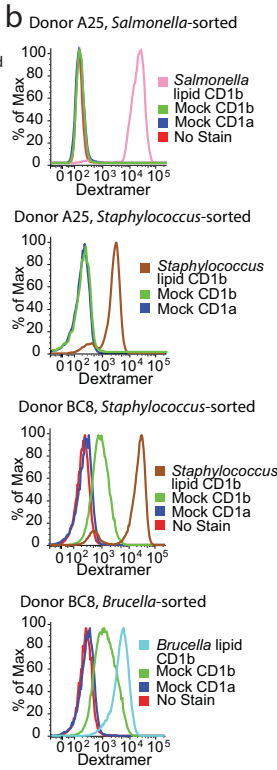
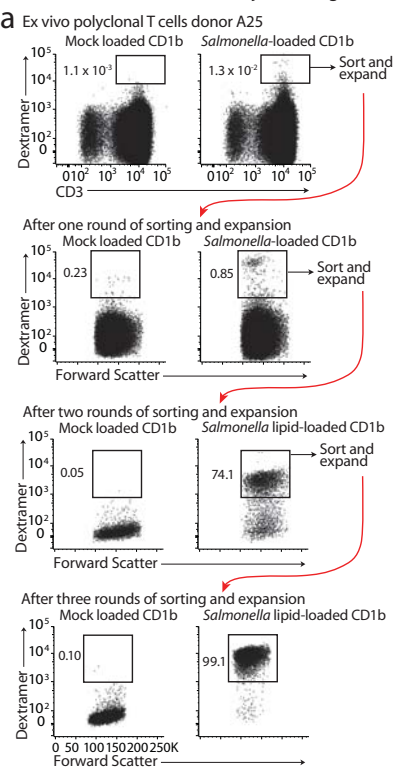
fifteen analytes was tested but not detected in response to C1R.CD1b or PMA and ionomycin: Interleukin-1 receptor antagonist (IL-1Ra), IL-5, IL-12, IL-21, IL-25, IFN- α , IFN- β , April, macrophage migration inhibitory factor (MIF), CCL17 (TARC), CXCL1 (Gro-1 α), soluble (s)PD-1, sFas, sFas-L, sTNF-RI. (c) The A25Salmonella T cell line was stimulated with CD1a or CD1b-transfected cells for 20 hours prior to flow cytometric analysis of CD25 or CD3 expression. In a, and c one representative experiment out of three is shown. For b, one experiment was performed on the pooled supernatants of triplicate wells. * P<0.05; ** P<0.01; NS: not significant.

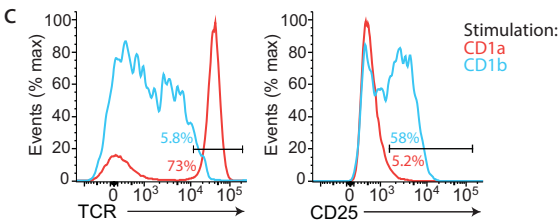
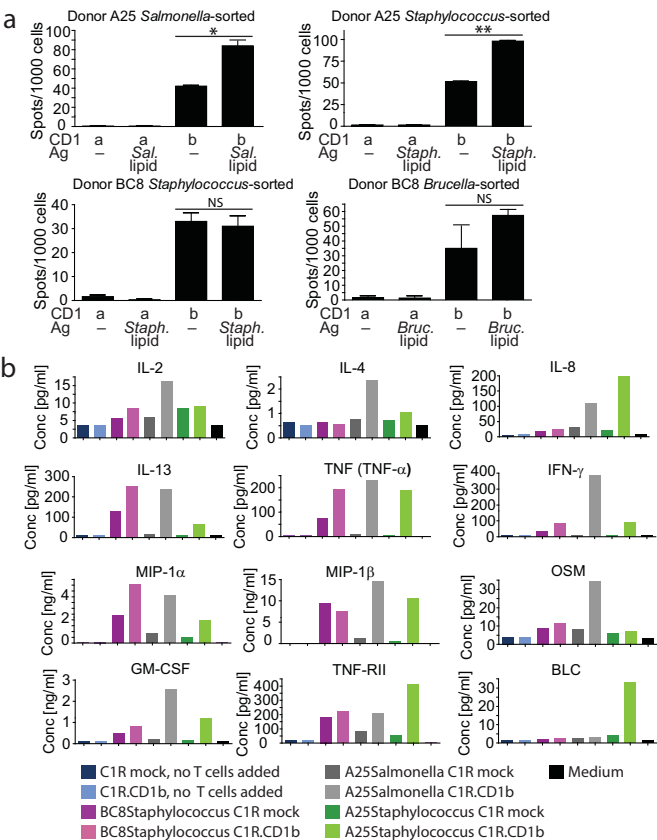
Fig. 3 Phosphatidylglycerol is a major target of CD1b-autoreactive T cells

(a) CD1b dextramers were loaded with the major classes of phospholipids and used to stain bacterial lipid-specific T cell lines. (b) Negative ions present in the methanol-eluted fractions from *S. aureus* and *S. Typhimurium* were analyzed by collisional mass spectrometry and generated patterns that are consistent with phosphatidylglycerol (PG). (c) The same two cell lines that were shown in a were analyzed using CD1b dextramers loaded with PG from mammalian and bacterial sources, the structures of which are shown in d. Panels a and c represent one of two independent experiments. Even though the branching pattern of fatty acids could not be determined directly, *S. aureus* fatty acids are drawn as anteiso forms because that is the most common form for C15 and C17 in this species (22). The C17 fatty acid in *S. Typhimurium* can be C17:1 or cyclopropyl C17 (38).

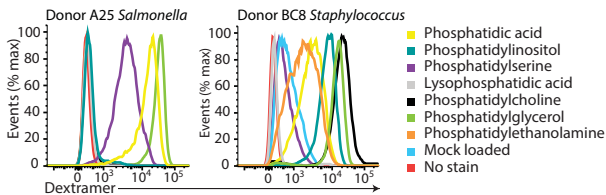
Fig. 4 Single CD1b autoreactive TCRs recognize multiple phospholipids

Two TCRs that were cloned from the A25Salmonella T cell line, named PG10 and PG90, were stably transduced into Jurkat cells. (a) The PG10 and PG90 TCR-expressing Jurkat cells were incubated overnight with wild type C1R cells or CD1b-transfected C1R cells before they were stained with an antibody against CD69. PG10 and PG90-expressing Jurkat cells were stained with CD1b tetramers loaded with the indicated lipids (b). Results in b represent one out of four independently performed experiments.

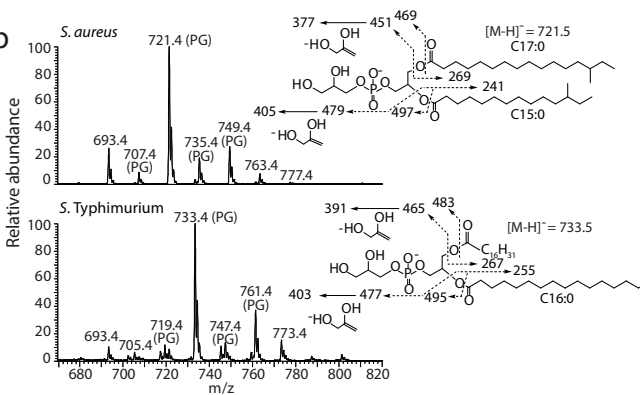




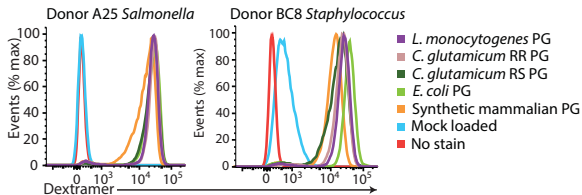
a



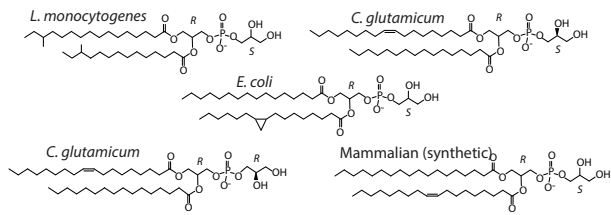
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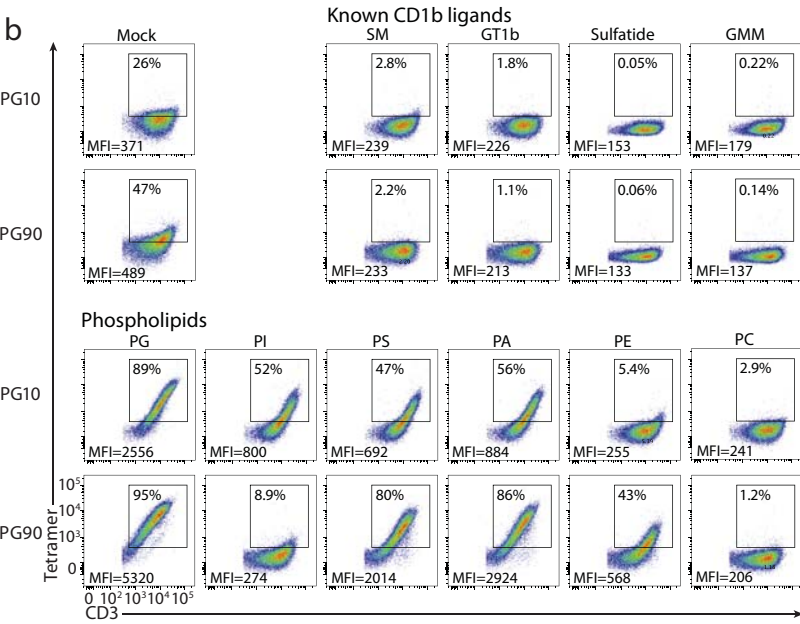
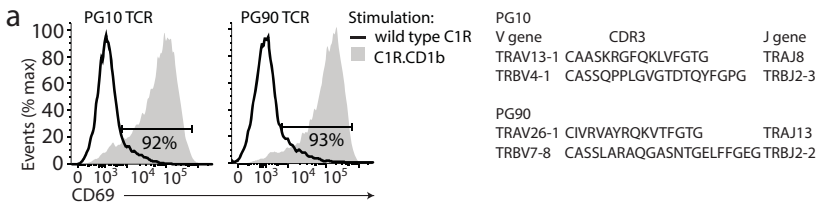
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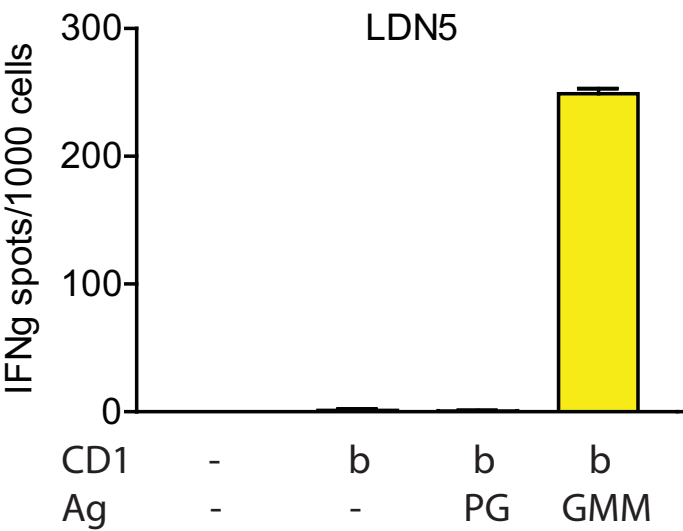
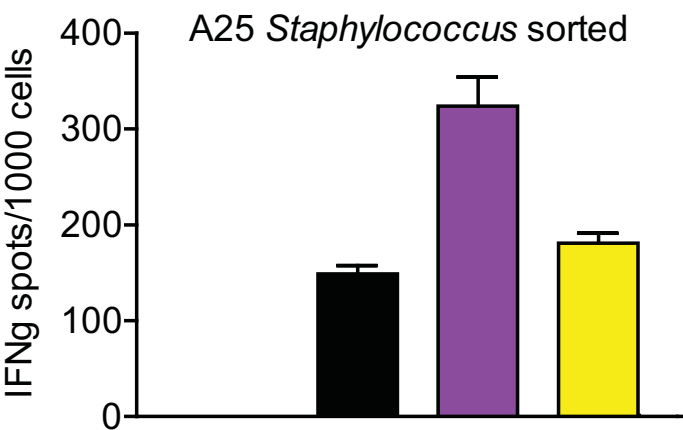
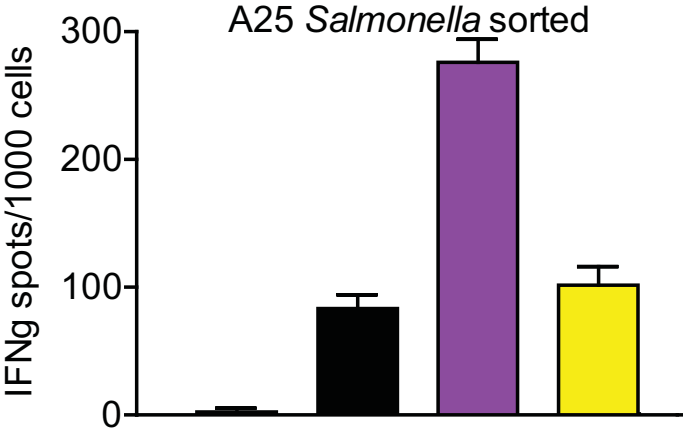


d



Van Rhijn et al. Figure 4





CD1

-

b

b

b

Ag

-

-

PG

GMM

