Benzofuran sulfonates and small self-lipid antigens activate type II NKT cells via CD1d

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Natural killer T (NKT) cells detect lipids presented by CD1d. Most studies focus on type I NKT cells that express semi-invariant αβ T cell receptors (TCR) and recognize α-galactosylceramides. However, CD1d also presents structurally distinct lipids to NKT cells expressing diverse TCRs (type II NKT cells), but our knowledge of the antigens for type II NKT cells is limited. An early study identified a nonlipidic NKT cell agonist, phenyl pentamethyldihydrobenzofuransulfonate (PPBF), which is notable for its similarity to common sulfa drugs, but its mechanism of NKT cell activation remained unknown. Here, we demonstrate that a range of pentamethylbenzofuransulfonates (PBFs), including PPBF, activate polyclonal type II NKT cells from human donors. Whereas these sulfa drug–like molecules might have acted pharmacologically on cells, here we demonstrate direct contact between TCRs and PBF-treated CD1d complexes. Further, PBF-treated CD1d tetramers identified type II NKT cell populations expressing αβTCRs and γδTCRs, including those with variable and joining region gene usage (TRAV12-1–TRAJ6) that was conserved across donors. By trapping a CD1d–type II NKT TCR complex for direct mass-spectrometric analysis, we detected molecules that allow the binding of CD1d to TCRs, finding that both selected PBF family members and short-chain sphingomyelin lipids are present in these complexes. Furthermore, the combination of PPBF and short-chain sphingomyelin enhances CD1d tetramer staining of PPBF-reactive T cell lines over either molecule alone. This study demonstrates that nonlipidic small molecules, which resemble sulfa drugs implicated in systemic hypersensitivity and drug allergy reactions, are targeted by a polyclonal population of type II NKT cells in a CD1d-restricted manner.

TCR | CD1d | antigen | PPBF | type II NKT

Natural killer T (NKT) cells are defined as T cells that are restricted to the lipid antigen-presenting molecule, CD1d. The most extensively studied are type I NKT cells, which typically express an invariant T cell receptor (TCR)-α chain consisting of TRAV10–TRAJ18 in humans (TRAV11–TRAJ18 in mice) paired with a constrained repertoire of TCR-β chains, enriched for TRBV25 in humans (TRBV13, 29, 1 in mice) (reviewed in ref. 1). Type I NKT cells are defined by their strong responses to α-galactosylceramide (α-GalCer) and structurally related hexosylceramides presented by CD1d, while in contrast, type II NKT cells are defined as CD1d-restricted T cells that express diverse TCRs and do not recognize α-GalCer (reviewed in refs. 1 and 2). Very little is known about the chemical identity of antigens for type II NKT cells; however, some studies suggest that these cells are abundant in humans (3–5), and, by virtue of their greater TCR diversity, they can interact with a broader range of antigens compared to type I NKT cells (2, 6–14).

In 2004, a nonlipidic molecule, phenyl pentamethyldihydrobenzofuransulfonate (PPBF), was described that stimulated a human TRAV10$^-$ (type II) NKT cell line (clone ABd) in a CD1d-dependent manner (15). These observations were notable because PPBF resembles various sulfonamide drugs: furosemide (diuretic), sulfasalazine (disease-modifying antirheumatic), and celecoxib (anti-inflammatory) as well as "sulfa" antibiotics such as sulfonamide, sulfapyridine, sulfamethoxazole, sulfadiazine, and sulfadoxine (16). These drugs can cause systemic delayed-type hypersensitivity reactions, which are thought to be mediated by T cells (17–20). Most of our limited understanding of drug hypersensitivity reactions comes from work focusing on Human Leukocyte Antigen (HLA)-restricted conventional T cells, which has led to the proposal of four main mechanisms for small-drug immune activity as reviewed in ref. 21: 1) hapten/prohapten formation, whereby the drug reacts with

Significance

Whereas T cells are known to recognize peptides, vitamin B metabolites, or lipid antigens, we identify several nonlipidic small molecules classified as pentamethylbenzofuransulfonates (PBFs) that activate a population of CD1d-restricted natural killer T (NKT) cells. This represents a breakthrough in the field of NKT cell biology. This study also reveals a previously unknown population of PBF-reactive NKT cells in healthy individuals with stereotyped receptors that paves the way for future studies of the role of these cells in immunity, including sulfa drug hypersensitivity.

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self-Ags to generate a neo-product that undergoes processing and presentation to T cells; 2) noncovalent/labile pharmacological interaction with immune receptors on the cell surface; 3) superantigen mediating direct linkage of TCRs and Ag-presenting molecules; and 4) anchor site occupation by small molecules in Ag-presenting molecules inducing an altered self-Ag repertoire (22). Whether the CD1d-NKT cell axis is implicated in drug hypersensitivity remains unclear (23). Whereas most antigens in the CD1d system are lipids that use their alkyl chains to bind to CD1d, PPBF is a polycyclic small molecule and so would have to act through an atypical display mechanism. As previous attempts to stain the ABd clone with CD1d-PPBF tetramers were unsuccessful, the atypical drug-like structure of PPBF raised the possibility of direct pharmacological action on T cells rather than the presentation of CD1d-PPBF complexes to TCRs. However, the mechanism of PPBF-mediated type II NKT cell activation remains undefined (15).

Here, using TCR-transduced cell lines, CD1d tetramers treated with PPBF, and new analogs in the pentamethylbenzofuransulfonate (PBF) family, we discovered that several molecules stimulate polyclonal NKT cells. Using CD1d tetramers treated with a newly identified and more potent analog of PPBF, we identified populations of type II NKT cells that comprise a polyclonal repertoire of both αβ and γδ T cells, including those with conserved TCR sequences. This enigmatic nature of T cell responses to PBF molecules was resolved using TCR trap technology (24, 25). Mass-spectrometric analysis of all molecules present in CD1d-PBF-TCR complexes indicates that CD1d binds PBFs and small self-lipids that promote CD1d-TCR binding. These data support a model of type II NKT cell recognition of small sulfa drug–like compounds in association with CD1d and flag a possible mechanism in which such cells may be involved in sulfa drug hypersensitivity.

Results

Role of the TCR in Recognition of PPBF Molecules. Previously, it was demonstrated that PPBF can stimulate a TRAV10^{-} αβ T cell clone, ABd, which was derived from polyclonal T cells expanded from human peripheral blood mononuclear cells (PBMCs) in the presence of PPBF (15). While the activation of ABd required CD1d (15), attempts to stain the T cell clone established in that study with PPBF-treated tetramers were unsuccessful. PPBF is a structurally unconventional stimulant of CD1d-reactive T cells, and these observations are consistent with either low-affinity binding of the TCR to PPBF-CD1d complexes or PPBF acting via a TCR-independent atypical mechanism, such as direct pharmacological activation of cells. We generated a TCR reporter cell line by transducing β2m-deficient SKW3 cells (SKW3.β2m$^{-/-}$, lacking endogenous CD1d) with the ABd TCR sequence (TRAV12-1–TRAJ6, TRBV11) to directly examine the role of the ABd TCR in PPBF responsiveness

Given the unusual, nonlipidic structure of PPBF, we studied the structural requirements for T cell recognition by using analogs of PPBF. Given that our previous work demonstrated that 3-methyl-PPBF was more potent than the 2- and 4-methyl isomers (15), we focused on substitutions at the phenyl 3 position. Using a one-step direct method for the synthesis of these molecules, we altered the methyl group to substituents varying in electron density (F, Cl, Br), steric bulk (isopropyl, iPr; *tert*-butyl, tBu; phenyl, Ph), and electron-donating/withdrawing ability (Me_2N, MeO, NO_2, CF_3) (*SI Appendix, SI Chemistry Experimental* and Fig 1*A*). Additional analogs included 2,3-dimethyl and 3,5-dichloro derivatives (DMePPBF and DClPPBF, respectively) to examine whether substituent effects are additive. We increased the ring size of the benzofuran to a benzochroman and studied linker variation by replacing the sulfonate with a sulfonamide (APBF, mAPBF, and MAPBF). We also included the unsubstituted cores: PBF, PBC, and a water-soluble analog, Trolox. These compounds were assessed for their ability to activate the ABd TCR–transduced cell line or a control type I human NKT TCR-expressing line (NKT15) (26) in cocultures with C1R Ag-presenting cells, which surface express very low levels of CD1d (*SI Appendix*, Fig. S4*A*). Measuring CD69 up-regulation as a readout for cellular activation, strong dose-dependent responses were detected to many of these analogs by the ABd cell line (Fig. 1*B*) but not by the NKT15 cell line, which responded only to α-GalCer.

Both compounds from our initial study (15), PPBF and 3-methyl PPBF (MePPBF), activated ABd TCR^{+} cells, eliciting a two- and threefold increase in CD69 mean fluorescence intensity (MFI), respectively, when used at 15 μM (Fig. 1*B*). This effect was largely lost when PPBF was used at 1.5 μM, but MePPBF was still active at this lower concentration, consistent with previous work that showed this analog was more potent than PPBF (15). This finding is notable because the ABd clone was derived from a culture in the presence of PPBF, yet the resulting T cell line responded better to MePPBF (Fig. 1*B* and ref. 15). Stronger responses than the unsubstituted parental PPBF were also elicited by NOPPBF, MeOPPBF, FPPBF, and, most notably, by BrPPBF and ClPPBF, which retained activity even at 0.15 μM. Conversely, PhPPBF, tBuPPBF, iPrPPBF, NMePPBF, and CF_3PPBF failed to induce responses, indicating a preference for a small group at the 3 position. The benzochroman MePPBC and the 3-chloro analog thereof 3-chlorophenyl 2,2,5,7,8-pentamethylbenzochromane-6-sulfonate (ClPPBC) were as potent as ClPPBF (*SI Appendix*, Fig. S1), whereas isomerization of the sulfonate linkage yielding the 3-methyl "isosulfonate" (IsoMePPBC) led to a loss of activity and binding (*SI Appendix*, Fig. S1), indicating the importance of linker orientation. The disubstituted compounds (DMePPBF and DClPPBF) did not elicit a response, highlighting the preference for substitution only at the 3 position. Finally, the sulfonamides APBF, mAPBF, MAPBF, and the unsubstituted core analogs PBF, PBC (a benzochroman), and Trolox did not induce a response, indicating that a sulfonate linker and the phenyl group are important for T cell activation (Fig. 1*B*).

Next, we challenged the ABd TCR-transduced cell line with PPBF, MePPBF, and α-GalCer in the presence of CD1d blocking or isotype control antibodies (Fig. 2*A*). PPBF and MePPBF stimulated the ABd TCR-transduced cells in a CD1d-dependent manner and were blocked by anti-CD1d antibody (α-CD1d) but not by an isotype control. PBF compounds did not activate the NKT15 type I NKT cell line, suggesting there was no general mitogenic effect. Conversely, NKT15 TCR-transduced cells were highly responsive to α-GalCer, and blocked by anti-CD1d, but were unresponsive to PPBF or MePPBF, demonstrating that the response to these compounds is TCR and CD1d dependent.

We also stained the ABd TCR-transduced cell line with CD1d tetramers treated with PPBF or two of the higher potency analogs (Figs. 1*B* and 2*B*). Similar to our previous work (15), CD1d-PPBF or CD1d-MePPBF tetramers failed to stain the ABd-TCR^{+} cell line above background with untreated CD1d tetramers (Fig. 2*B*). However, ClPPBF, BrPPBF, or ClPPBC treatment enabled CD1d tetramer staining of CD3/TCR^{hi} cells (Fig. 2 and *SI Appendix*, Fig. S1). Conversely, CD1d tetramers loaded with α-GalCer stained the control NKT15 type I NKT cell line but not the ABd type II NKT cell line (Fig. 2*B*). These data can potentially be explained by the "affinity–avidity gap," whereby certain molecules generate complexes with MHC or CD1 proteins to which the TCRs bind with sufficient affinity to functionally activate T cells but do not mediate tetramer binding to TCRs (27–30). Accordingly, the two less potent agonists, PPBF and MePPBF, appear to fall within the affinity–avidity gap, whereas ClPPBF, ClPPBC, and BrPPBF induce the formation of higher avidity CD1d complexes that permit tetramer staining. Thus, the ABd type II TCR directly binds CD1d and does so in a PBF-dependent manner. Interestingly, the PBF and PBC analogs used in this study failed to activate or impact on CD1d tetramer binding of other type II NKT TCR-transduced cell lines (CD1d-endo reactive clones T14, VD3G8, T26B; *SI Appendix*, Fig. S2), suggesting that PBFs are not general type II NKT cell agonists or T cell mitogens.

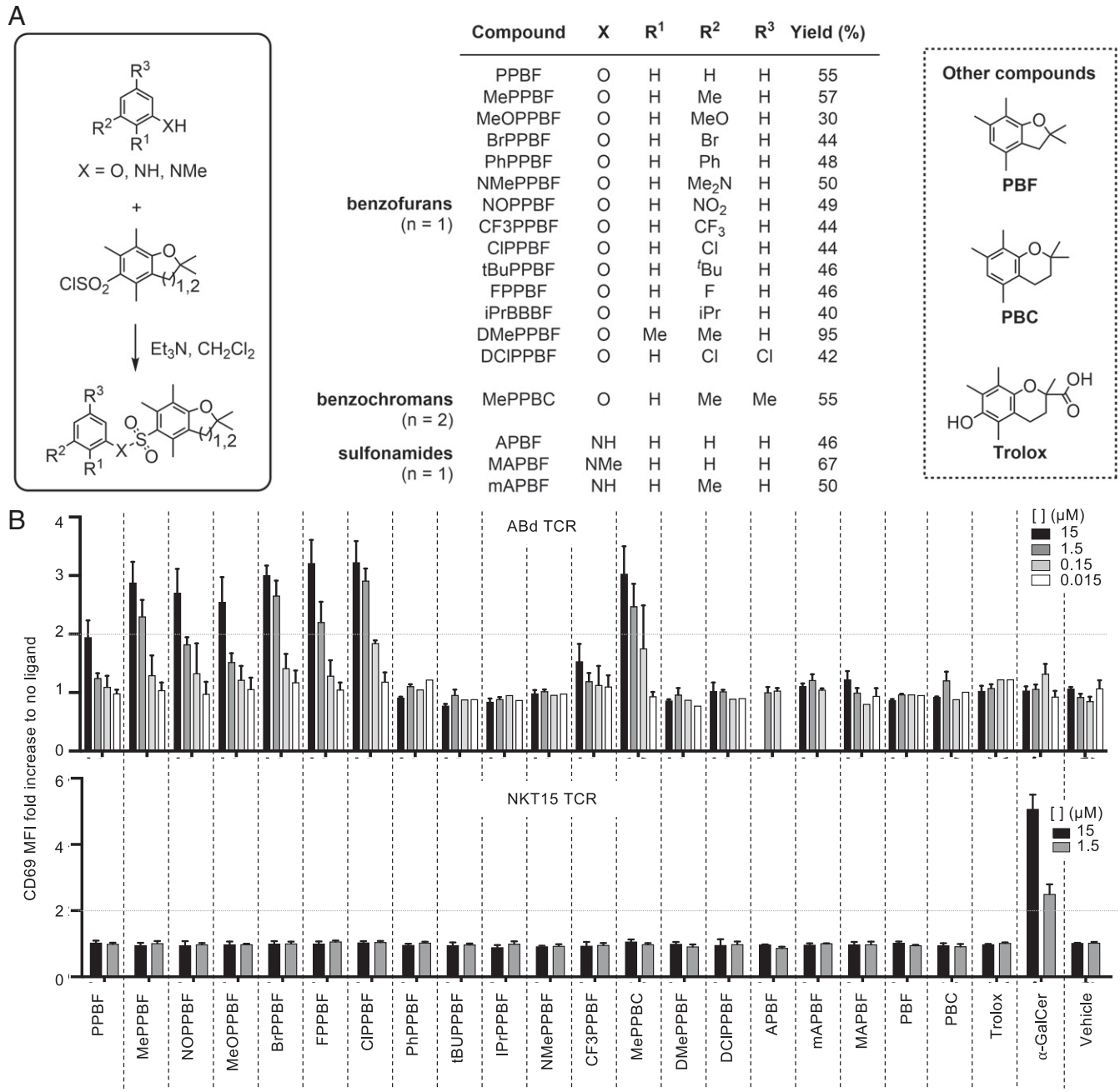

Fig. 1. Structure activity relationship study of PPBF analogs on ABd type II NKT TCR$^+$ cells. (*A*) Synthesis of various PPBF analogs, including modifications at positions 2′ and 3′ of the phenyl ring, the sulfonate linkage, and ring expansion of the benzofuran. Yield (%) values depict chemical reaction yields. (*B*) Activation of a SKW3.β2m$^{-/-}$ cell line stably transduced with the ABd type II NKT TCR [PPBF reactive (15)] when cocultured with C1R antigen-presenting cells and serially diluted (10-fold dilutions ranging 15 to 0.015 μM) PPBF analogs (from *A*) for 16 h—without washing out the ligand. α-GalCer and the NKT15 type I NKT TCR$^+$ cell line [α-GalCer reactive (26)]. Graphs depict fold increase of CD69 up-regulation in comparison to conditions with no ligand after 16 h activation. The dotted horizontal line marks a twofold increase over no ligand control. Data are from three independent experiments + SEM.

Identification of CD1d-PPBF–Reactive NKT Cells in PBMCs. Having established that CD1d-endo tetramers treated with some PBF analogs enable staining of the ABd TCR$^+$ SKW3.β2m$^{-/-}$ cells, we used CD1d-ClPPBF tetramers to identify PBF-reactive cells in PBMCs from healthy human blood donors. Compared to the frequency of staining of type I NKT cells with CD1d-α-GalCer tetramers among CD3$^+$CD14$^-$CD19$^-$7AAD$^-$ human lymphocytes (0.079 to 0.23%), ClPPBF-treated CD1d tetramers stained larger numbers (~1%) of lymphocytes but with much lower MFI (Fig. 3*A*). To allow functional testing of TCRs and CD1d recognition, we enriched CD1d-ClPPBF tetramer$^+$ cells using tetramer-associated magnetic enrichment (TAME) (31). One round of TAME with Phycoerythrin (PE)-labeled CD1d-ClPPBF tetramers increased the frequency of the CD1d-ClPPBF tetramer$^+$ among CD3$^+$ T cells (6 to 15%) and yielded more defined clusters of brightly stained populations in most cases, in contrast to Phycoerythrin-conjugated streptavidin (SAV-PE) controls. Unexpectedly, in most donors, these cells included both αβ T cells (CD3$^+$γδTCR$^-$) and γδ T cells (CD3$^+$γδTCR$^+$) cells, identifying the first γδ T cells to recognize PBF-treated CD1d, which, in donor C3, represented the majority of CD1d-ClPPBF tetramer$^+$ cells.

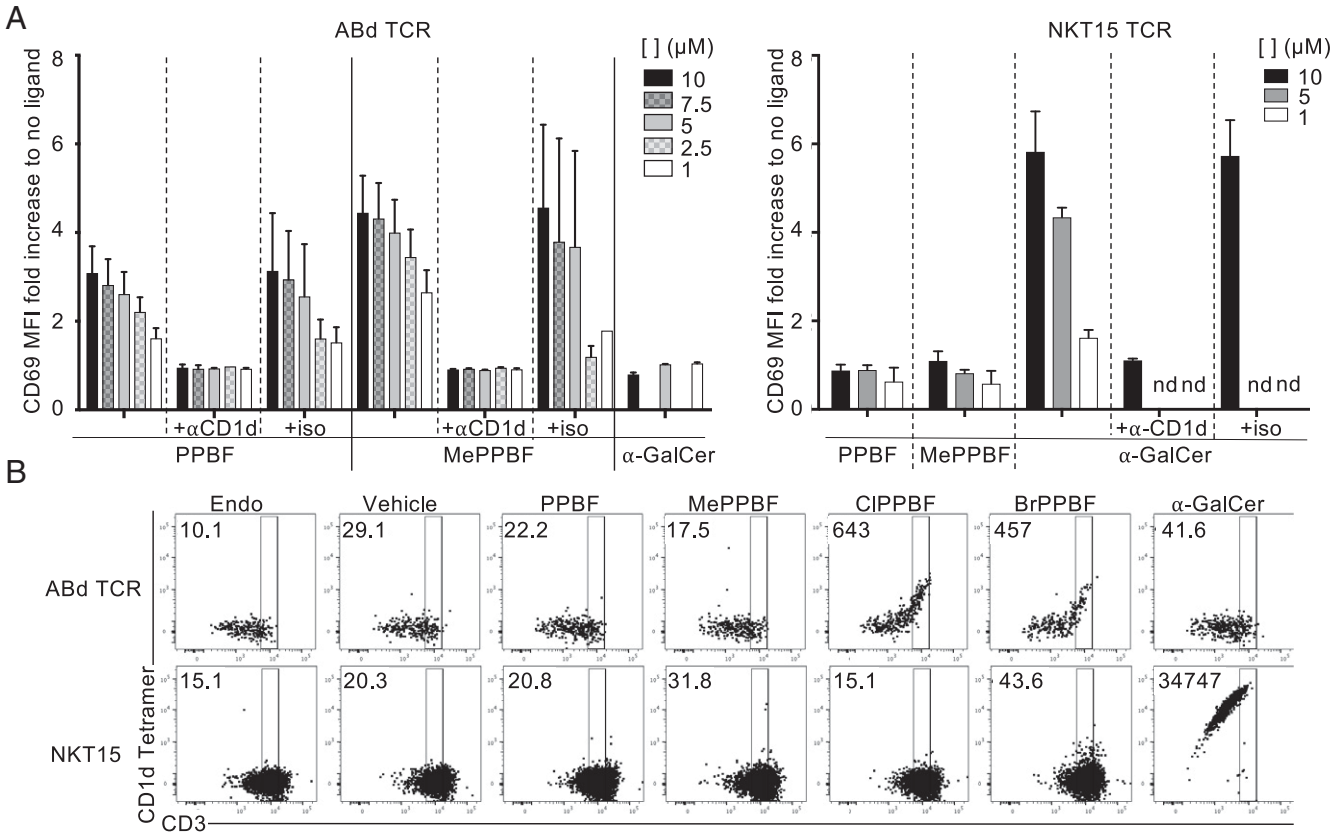

Fig. 2. PPBF-mediated type II NKT cell activation is due to TCR recognition of CD1d-PPBF complexes. (*A*) PPBF or MePPBF at concentrations ranging from 1 to 10 μM were added to cocultures of C1R cells and a SKW3.β2m$^{-/-}$cell line stably transduced with the ABd type II NKT TCR in the presence or absence of α-CD1d–blocking antibody or isotype control (without washing out excess ligand). α-GalCer and the NKT15 type I NKT TCR$^+$ cell line were included as controls. Graphs depict fold increase of CD69 up-regulation in TCR-transduced SKW3.β2m$^{-/-}$cell lines in comparison to conditions with no ligand after 16 h activation. Data are from three independent experiments + SEM. (*B*) Biotinylated CD1d monomers (10 μM) were incubated overnight at pH 6 to 6.5 4 °C with candidate PPBF analogs (200 μM), tetramerized using SAV-PE, and assessed for their ability to stain the ABd type II NKT TCR$^+$ cell line by flow cytometry, without removal of unbound ligands. α-GalCer–loaded or "unloaded" (endo)-CD1d tetramer and the NKT15 type I NKT TCR$^+$ cell line were included as controls. Artwork shows representative dot plots from $n > 4$ experiments performed and the MFI of tetramer staining of cells with similar TCR levels.

Flow cytometric cell sorting of CD1d-ClPPBF tetramer$^+$ cells, followed by in vitro expansion in the presence of anti-CD3 and anti-CD28 plus cytokines (IL-2, IL-7, and IL-15) for 21 d, yielded large CD1d-ClPPBF tetramer$^+$ T cell populations (~0.3 to 30% of CD3$^+$ cells) from all donors tested (Fig. 3*B*). More so than presort staining, these enriched and expanded cells exhibited clear CD1d-ClPPBF tetramer staining, which correlated with CD3 expression levels as expected when tetramers bind TCRs. Similar to ex vivo analysis of nonexpanded CD1d-ClPPBF-tetramer binding populations (Fig. 3*A*), in vitro–expanded populations included both αβ T cells and γδ T cells in variable proportions. Most CD1d-ClPPBF tetramer$^+$ αβ T cell clones were CD4$^+$ or CD8$^+$, whereas γδ T cells that bound the CD1d-ClPPBF tetramer were CD4$^-$CD8$^-$ double negative (DN), typical of γδ T cells, including those described to interact with CD1d (32–34). Thus, human PMBCs from healthy donors contain populations of αβTCR$^+$ and γδTCR$^+$ cells that can recognize CD1d treated with nonlipidic PBF molecules.

The above data could not distinguish between T cells that were reactive to CD1d alone from those that specifically react with CD1d in association with PBF antigen. To investigate this further, in vitro–expanded tetramer$^+$ cells were dual stained with CD1d-ClPPBF tetramers and CD1d tetramers that were not treated with ClPPBF but likely still carry endogenous lipids ("CD1d-endo") derived from the mammalian expression system. In all subjects (four shown, representative of 13 donors analyzed), the two-dimensional staining pattern showed several distinct T cell clusters, which likely represent oligoclonal expanded subpopulations of NKT cells (Fig. 3*C*). Most clusters costained with CD1d-endo and CD1d-ClPPBF and displayed a positive correlation for staining intensity on a diagonal, suggesting both tetramers bound the same TCR. However, in some cases, CD1d-ClPPBF tetramers stained more brightly relative to CD1d-endo tetramers, while in other cases, the opposite was observed, and both examples were seen even within the same donor. Thus, the presence of ClPPBF may modulate the recognition of some self (endo) lipids present in CD1d tetramers, or CD1d itself, by some NKT cells.

Diverse but Biased CD1d-ClPPBF Tetramer$^+$ NKT TCRs. To dissect the molecular basis of CD1d-PBF TCR reactivity, we sought to clone αβTCRs and γδTCRs from CD1d-ClPPBF–reactive T cells, generate TCR reporter cell lines, and measure their interactions with CD1d, self-lipids, ClPPBF, and α-GalCer. Using dual tetramer staining, we sorted single cells from the upper left quadrant that were stained with CD1d-ClPPBF tetramer at higher levels than by CD1d-endo tetramers (Fig. 3*C*) from 13 blood donors for multiplex single-cell TCR sequencing (35) to identify 47 paired αβTCR sequences and 16 paired γδTCR sequences (Table 1 and Fig. 3*D*). None of the sorted cells expressed the defining *TRAV10–TRAJ18* rearrangement for human type I NKT cells. Of the 16 unique paired γδTCR sequences and four unpaired sequences (not included),

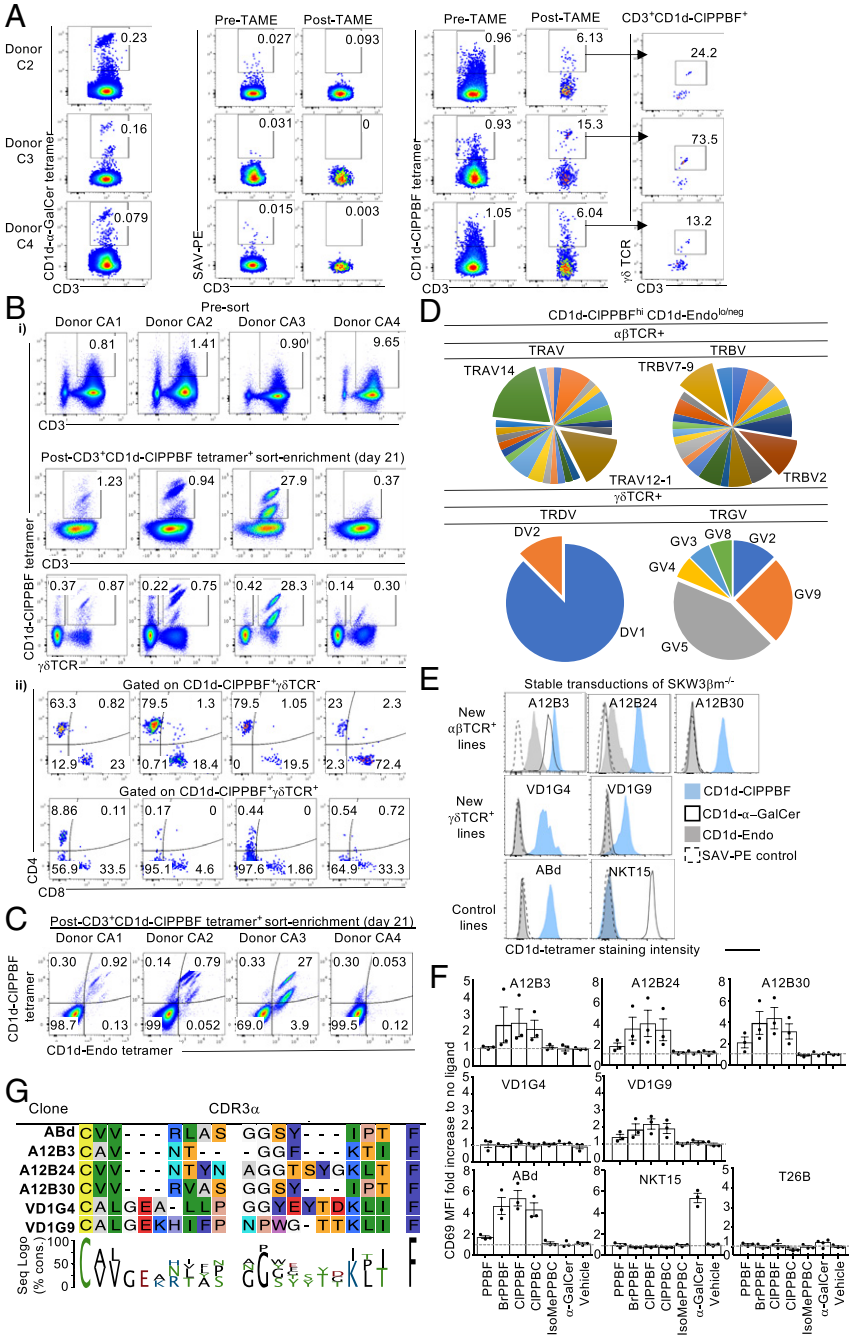

Fig. 3. Identification of human CD1d-ClPPBF tetramer$^+$ T cells in PBMCs. (*A*) Identification of CD1d–α-GalCer tetramer$^+$ (type I NKT) (*Left*) or CD1d–ClPPBF tetramer$^+$ cells in PBMCs (*Right*). Flow cytometry plots of three donors showing CD3 versus CD1d–α-GalCer tetramer (*Left*) or SAV-PE and CD1d–ClPPBF tetramer staining (*Right*) pre– and post– CD1d-ClPPBF TAME on gated 7AAD$^-$CD14$^-$CD19$^-$ single lymphocytes. CD3 versus γδTCR stain is shown for CD3$^+$CD1d–ClPPBF tetramer$^+$ cells post-TAME. Data are representative of $n = 10$ individual donors across three independent experiments. (*B*) CD1d-ClPPBF$^+$CD3$^+$ cells were bulk sorted and expanded in vitro for 21 d with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of IL-2, IL-7, and IL-15. After expansion, samples were reassessed for their ability to bind CD1d-ClPPBF tetramers. Representative plots of four donors showing pre- and postsort-enriched CD3 or γδTCR versus CD1d–ClPPBF tetramer staining (*i*). CD4 versus CD8 expression on αβ T cells (CD3$^+$CD1d$^-$ClPPBF$^+$γδTCR$^-$) or γδ T cells (CD3$^+$CD1d$^-$ClPPBF$^+$γδTCR$^-$) (*ii*). Plots are representative of $n = 13$ individual donors across six independent experiments. (*C*) Dual tetramer labeling showing CD1d-endo versus CD1d–ClPPBF tetramers on sort-enriched cells gated for 7AAD$^-$CD19$^-$CD14$^-$CD3$^+$ cells. (*D*) Single positive CD1d-ClPPBF$^+$CD1d-Endo$^-$CD3$^+$ cells were single-cell sorted into individual wells for TCR gene PCR amplification. Pie charts with TCR genes used were derived from analysis of 13 donors (αβTCRs) or seven donors (γδTCRs) of six independent sorting experiments. (*E*) SKW3.β2m$^{-/-}$ cells were stably transduced with TCR sequences for which CD1d reactivity had been validated in *SI Appendix*, Fig. S1 and stained with anti-CD3 antibody and ClPPBF-, α-GalCer–loaded or "unloaded" (endo)-loaded CD1d tetramers. Histograms show tetramer staining of cells previously gated for a confined range of TCR expression levels to ensure that different TCR expression between cell lines was not a variable and are representative of $n = 3$ independent experiments. SAV-PE control staining was also included (dashed line). (*F*) NKT TCR-transduced SKW3.β2m$^{-/-}$ cell lines were cocultured for 16 h with THP-1 cells in the presence of PPBF, BrPPBF, ClPPBF, ClPPBC, IsoMePPBC, or α-GalCer at 10 μM. The T26B and NKT15 cell lines were included as controls. Graphs show the level of activation (CD69-fold increase) in comparison to no ligand. Data are from $n = 3$ independent experiments + SEM. (*G*) Alignment of the CDR3-α/δ loops and Sequence Logo showing percent conservation (cons.) using CLC Main Workbench 7.

13 displayed *TRDV1* rearrangements consistent with previous studies that described the same bias among CD1d-restricted γδTCR$^+$ (32–34) or δ/αβTCR (36) T cells. Among the αβ T cells, *TRAV14* was observed in nine paired and two unpaired (not included) TCR-α sequences, and *TRAV12*$^+$ genes were overrepresented with *TRAV12-1* present in seven clones with paired sequences and in three with unpaired TCR-α sequences (not included), *TRAV12-2*, in two sequences, and *TRAV12-3* in one. Indeed, TRAV12-1, TRAV14, and TRDV1 TCRs feature conserved serines within their CDR1-α/δ(Ser_{28} and Ser_{36}) and CDR2-α/δ (Ser_{58}), which may reflect TRAV/TRDV gene-encoded contributions to CD1d-PPBF specificity and associated bias (Table 1 and Fig. 3*D*). Among the *TRAJ* genes, *TRAJ6* and *TRAJ52* were preferred, with TRAV12$^+$ TCRs preferentially pairing with *TRAJ* genes that confer a GG pattern to the CDR3-α loop (Table 1A). Furthermore, three out of seven TRAV12-1$^+$ TCRs preferentially rearranged with *TRAJ6* and five out of seven TRAV12-1$^+$ clones generated CDR3-α loops with a conserved length of 15 amino acids. These variable and joining region gene patterns are notable because they were detected across different donors, including the ABd TCR, which was isolated many years earlier in a different study (15), and also shared the exact same CDR3-α loop as a clone identified in our new study from a different donor (Table 1A, fourth donor from the top). These results suggest an interdonor TCR-α chain conservation among human PPBF-reactive type II NKT cells, albeit a less strict conservation than seen in type I NKT cells (Table 1). Together, this suggests a role for CDR3-α in determining the specificity of NKT TCRs for CD1d with PPBF. Within the TCR β-chain rearrangements, there was a preference for *TRBV7-9* and *TRBV2* gene segments, which differed to the preference for *TRBV25* by type I NKT cells (reviewed in ref. 1). Together, these data suggest that CD1d-ClPPBF tetramers identify a population of T cells expressing diverse but moderately biased αβTCRs (*TRAV12* and *TRAV14*) or *TRDV1*-biased γδTCRs and that these are distinct from type I NKT TCRs.

Specificity of the CD1d-ClPPBF Tetramer$^+$ T Cells. To test the specificity of the putative CD1d-ClPPBF–reactive TCRs, we selected nine αβTCR and two γδTCR sequences from seven donors (Table 1) of which three TRAV12$^+$ αβTCRs (clones A12B3, A12B24, and A12B30) and both γδTCR sequences (clones VD1G9 and VD1G4) recapitulated CD1d-ClPPBF tetramer staining using human embryonic kidney (HEK) 293T-based TCR transfectants (*SI Appendix*, Fig. S3). These validated TCR sequences were stably transduced into SKW3.β2m$^{-/-}$ clones and assessed for binding to CD1d-ClPPBF, CD1d-endo, or CD1d-α-GalCer tetramers (Fig. 3*E*) along with the original PPBF-reactive ABd TCR and type I NKT TCR cell lines, which served as controls for this experiment. The four TRAV12$^+$ clones, including ABd as well as the two γδTCR clones, were stained strongly by CD1d-ClPPBF tetramers at levels above CD1d-endo tetramers or the SAV control (Fig. 3*E*). Despite that, some clones were also stained by CD1d-endo tetramers above SAV control (*SI Appendix*, Fig. S3 and Fig. 3*E*), showing that PBFs increase CD1d-TCR binding but are not absolutely required for CD1d-TCR interaction. Conversely, the NKT15 TCR cell line was only stained by CD1d-α-GalCer tetramers. Interestingly, α-GalCer–loaded CD1d tetramers also stained the A12B3 clone more brightly than CD1d-endo but not as brightly as CD1d-ClPPBF tetramers, suggesting that this is an atypical NKT TCR whose CD1d binding is also enhanced by the presence of α-GalCer, similar to our previous reports (37–39). Next, we investigated whether these newly identified T cell clones were activated in response to a small panel of PPBF analogs using THP-1 antigen-presenting cells, which surface express moderate levels of CD1d (*SI Appendix*, Fig. S4). Additionally, the type I NKT15 TCR and the CD1d-endo reactive type II T26B TCR cell lines were included as controls. ClPPBF, BrPPBF, and ClPPBC elicited clear responses by all TRAV12$^+$ clones and the VD1G9$^+$ clone (Fig. 3*F*). The VD1G4 clone, the control type I NKT15 clone, and an unrelated type II clone (T26B) were not activated by these PPBF analogs. As expected, IsoMePPBC failed to activate any of these clones, while the NKT15 TCR cell line only responded to α-GalCer. These results suggest that these newly identified ClPPBF-reactive clones display a similar preference for PPBF analogs with 3′-substituted phenyloxysulfonyl motif, including orientation of the sulfonate linkage and permissiveness to expansion of the benzofuran ring. Despite the variable lengths within their CDR3-α loops, six out of seven TCR sequences that were validated to have preferential PBF reactivity shared conserved GG amino acid motifs within their CDR3-α loop (Table 1 and Fig. 3*G*). Taken together, we have defined a previously unknown population of CD1d-restricted T cells that express PBF-reactive TCRs in the blood of healthy human donors.

Potential Docking Mode of PPBF-Reactive TCRs. Next, we investigated the CD1d docking mode of PPBF-reactive αβ and γδ TCRs using a panel of C1R cell lines transduced to overexpress wild-type CD1d (WT C1R.CD1d) or a series of CD1d alanine-substituted mutants on solvent-exposed residues of CD1d, where TCRs have been previously shown to bind (32, 40) (*SI Appendix*, Fig. S4 *A* and *B*). With the exception of the Val72 CD1d mutant, which only surface expressed 75% of the CD1d MFI when compared to WT C1R.CD1d, all other mutant cell lines displayed similar CD1d surface expression (<25% variation to WT C1R.CD1d) (*SI Appendix*, Fig. S4*B*). C1R.β2m$^{-/-}$ cells (which lack CD1d; *SI Appendix*, Fig. S4*A*) were also generated as controls. Interestingly, the overexpression of CD1d in this system was sufficient to elicit activation on all TCR-transduced cells except the VD1G4 clone. As expected, the addition of ClPPBF to the cocultures with C1R.CD1d led to a further increase in activation of all PPBF-reactive TCR reporter cell lines, including the VD1G4 clone but not the control type I and type II NKT cell lines (NKT15 and T26B) (*SI Appendix*, Fig. S4*B*). Conversely, the ClPPBF addition to cocultures with C1R.β2m$^{-/-}$ failed to elicit activation on the PPBF-reactive clones. The docking footprint of NKT15 TCR-CD1d-α-GalCer is known from a ternary crystal structure (40) and was used as a reference to interpret the effects of CD1d mutation in response to CD1d-transduced C1R cells. As expected, CD1d mutations affecting residues involved in binding of the type I NKT TCR to CD1d-α-GalCer such as Glu83-Ala, Val147-Ala, Lys86-Ala, and Met87-Ala, which form a ring around the F′ antigen portal, had a moderate (orange) or severe (red) impact on CD69 upregulation of the NKT15 cell line (Fig. 4).

The cellular activation of all PBF-reactive TCR$^+$ cell lines was severely impacted by the mutation of Trp160, which sits in the central A′ roof of the α2 helix of CD1d. Trp160 had previously been reported to mediate several interactions with atypical α-GalCer reactive TRAV10–TRAJ18$^-$ TCRs (38) via Tyr32-α and Gln31-α. Interestingly, Gln31-α is conserved among TRAV12-1, TRAV12-2, TRAV12-3, and TRAV14 TCRs (Table 1). The Trp160 mutation did, however, not affect the activation of the NKT15 TCR, again consistent with distinct binding footprints on CD1d. ABd activation was reduced by mutations in the A′ to F′ side of CD1d (Val72, Arg79, His68, Lys86, Val147, and Met87), which suggests that this TCR docks centrally on CD1d, and its footprint overlaps with the antigen portal. Interestingly, the His68-Ala mutation enhanced activation in the absence but not in the presence of ClPPBF. In the presence of the ligand, some of the effects of these CD1d mutations were lost or reduced, suggesting that the ligand may stabilize binding. This was also seen for other PPBF-reactive TCRs, albeit to a lesser extent. The impact of CD1d mutagenesis on the recognition of the other TCRs was consistent with similar but not identical footprints. Thus, A12B3 and VD1G4 activation was also affected by mutations in the same residues around the A′ side of the antigen portal (His68, Val72, and Thr157), whereas A12B24 was affected by mutations more central to the antigen portal

Table 1. Paired TCR sequences of CD1d-ClPPBF tetramer$^+$ single-sorted cells A

			TCRα						TCRβ						
Clone	Donor	Sorted as	TRAV	TRAJ	CDR1α	CDR2α	CDR3α		TRBV	TRBJ	TRBD	CDR1β	CDR2β	CDR3β	Obs
	CA1	ClPPBF+ Endo-	TRAV12-1*01	TRAJ6*01	NSA.....SQS	VYS.....SGN	CVV	XX ASGGSYIPTF	TRBV7-9*07	TRBJ2-2*01	TRBD2*02	SEH.....NR	FQN....EAQ	CAS EEGERIY	TGELFF 2
	KG 29	ClPPBF+ Endo-	TRAV12-1*01	TRAJ10*01	NSA.....SQS	VYS.....SGN	CVV	RF LTGGGNKLTF	TRBV7-9*01	TRBJ2-1*01	TRBD1*01 F	SEH.....NR	FQN....EAQ	CAS GDSQKWD	SYNEQFF 4
	CA1	ClPPBF+ Endo-	TRAV12-1*01	TRAJ52*01	NSA.....SQS	VYS.....SGN	CVVN	FA NAGGTSYGKLTF	TRBV30*01	TRBJ2-6*01	TRBD1*01	GTS.....NPN	SVG.....IG	CAW RKTDFS	SGANVLTF 2
	CA1	ClPPBF+ Endo-	TRAV12-1*01	TRAJ6*01	NSA.....SQS	VYS.....SGN	CVV	RL ASGGSYIPTF	TRBV2*01	TRBJ2-3*01	TRBD1*01	SNH.....LY	FYN.....NEI	CAS N	QY 4
A12B24	CA2	ClPPBF+ Endo-	TRAV12-1*01	TRAJ52*01	NSA.....SQS	VYS.....SGN	CVVN	TY NAGGTSYGKLTF	TRBV24-1*01	TRBJ2-6*01	TRBD1*01	KGH.....DR	SFD.....VKD	CATS GTDY	SGANVLTF 6
A12B9	CA3	ClPPBF+ Endo-	TRAV12-1*01	TRAJ5*01	NSA.....SQS	VYS.....SGN	CVV	IVM DTGRRALTF	TRBV9*01	TRBJ2-1*01	TRBD1*01	SGD.....LS	YYN....GEE	CASS QTPGTSS	SYNEQFF 8
A12B30	KG 28	ClPPBF+ Endo-	TRAV12-1*01	TRAJ6*01	NSA.....SQS	VYS.....SGN	CVV	RV ASGGSYIPTF	TRBV30*01	TRBJ1-1*01	TRBD1*01 F	GTS.....NPN	SVG.....IG	CAW RFQK	NTEAF 5
A12B3	CA3	ClPPBF+ Endo-	TRAV12-2*01	TRAJ9*01	DRG.....SQS	IYS.....NGD	CAVN	TGGFKTIF	TRBV3-1*01	TRBJ1-1*01	TRBD2*01	LGH.....DT	YNN....KEL	CASS RGLSWRGASFT	NTEAFF 7
	KG 30	ClPPBF+ Endo-	TRAV12-2*01	TRAJ23*01	DRG.....SQS	IYS.....NGD	CAV	QGGKLIF	TRBV20-1*01	TRBJ2-1*01	TRBD2*02 F	DFQ.....ATT	SNEG..SKA	CS STSGSLD	EQFF 1
	KG 27	ClPPBF+ Endo-	TRAV12-3*01	TRAJ24*01	NSA.....FQY	TYS.....SGN	CAM	TTDSWGKLQF	TRBV19*01	TRBJ2-3*01	TRBD2*01 F	LNH.....DA	SQI.....VND	CASSI DPTGAGVS	DTQYF 1
	KG 27	ClPPBF+ Endo-	TRAV14*01	TRAJ4*01 F	TSDP....SYG	QGSY..DQQN	CAMRE	G FSGGYNKLIF	TRBV2*01 F	TRBJ2-7*01	TRBD2*01 F	SNH.....LY	FYN.....NEI	CA TSGGLDK	NEQYF 2
	4D	ClPPBF+ Endo-	TRAV14*01	TRAJ40*01	TSDP....SYG	QGSY..DQQN	CAM	SD SGTYKYIF	TRBV20-1*01	TRBJ2-1*01	TRBD2*01	DFQ.....ATT	SNEG..SKA	CSAR DSVERG	SYNEQFF 2
	4D	ClPPBF+ Endo-	TRAV14*01	TRAJ52*01	TSDP....SYG	QGSY..DQQN	CAMRE	GD AGGTSYGKLTF	TRBV13*01	TRBJ2-3*01	TRBD2*02	PRH.....DT	FYE....KMQ	CASSL G	DTQYF 1
	CH8	ClPPBF+ Endo-	TRAV14*02	TRAJ42*01	TSDQ....SYG	QGSY..DEQN	CAMRE	L NYGGSQGNLIF	TRBV28*01	TRBJ2-7*01	TRBD2*01	MDH.....EN	SYD....VKM	CASL PRGVIH	2
T14	C3	ClPPBF+	TRAV14*02	TRAJ34*01	TSDQ....SYG	QGSY..DEQN	CAMR	AFD NTDKLIF	TRBV19*01	TRBJ1-2*01	TRBD2*01	LNH.....DA	SQI.....VND	CASS MAWRP	YGYTF 1
A14B7	CA1	ClPPBF+ Endo-	TRAV14*02	TRAJ3*01	TSDQ....SYG	QGSY..DEQN	CAMRE	DL YSSASKIIF	TRBV7-9*01	TRBJ1-1*01	TRBD1*01	SEH.....NR	FQN.....EAQ	CASSL G	EAFF 7
	CA1	ClPPBF+ Endo-	TRAV14*02	TRAJ17*01	TSDQ....SYG	QGSY..DEQN	CAMRE	GL AAGNKLTF	TRBV24-1*01	TRBJ2-2*01	TRBD1*01	KGH.....DR	SFD....VKD	CATSD TLA	GELFF 1
	CA2	ClPPBF+ Endo-	TRAV14*02	TRAJ56*01	TSDQ....SYG	QGSY..DEQN	CAMRE	AP TGANSKLTF	TRBV6-5*01	TRBJ1-6*01	TRBD1*01	MNH.....EY	SVG.....AGI	CASS LP	YNSPLHF 1
	KG 29	ClPPBF+ Endo-	TRAV14*02	TRAJ5*01	TSDQ....SYG	QGSY..DEQN	CAMR	DTGRRALTF	TRBV7-9*01	TRBJ2-1*01	TRBD1*01 F	SEH.....NR	FQN....EAQ	CASSL VGGPG	EQFF 1
	CH8	ClPPBF+ Endo-	TRAV17*01	TRAJ57*01	TSI.....NN	IRSN.....ERE	CATD	PP AQGGSEKLVF	TRBV7-9*01	TRBJ2-7*01	TRBD2*01	SEH.....NR	FQN....EAQ	CASSL E	EQYF 1
	CH8	ClPPBF+ Endo-	TRAV17*01	TRAJ54*01	TSI.....NN	IRSN.....ERE	CATD	DG GAQKLVF	TRBV27*01	TRBJ1-1*01	TRBD1*01	MNH.....EY	SMN....VEV	CASSL SLR	NTEAFF 1
A17B15	KG29	ClPPBF+ Endo-	TRAV17*01 F	TRAJ48*01 F	TSI.....NN	IRSN.....ERE	CAT	ER NFGNEKLTF	TRBV15*02 F	TRBJ2-5*01	TRBD1*01 F	LNH.....NV	YYD....KDF	CAT SETGG	TQYF 3
	CA4	ClPPBF+ Endo-	TRAV19*01	TRAJ22*01	TRDT.....TYY	RNSF...DEQN	CALSE	AR GSARQLTF	TRBV9*01	TRBJ1-4*01	TRBD1*01	SGD.....LS	YYN....GEE	CASS VGGQGY	EKLFF 2
	CH8	ClPPBF+ Endo-	TRAV20*01	TRAJ24*02	VSG.....LRG	LYSA.....GEE	CAVQ	AG DSWGKLQF	TRBV11-2	TRBJ2-1*01	TRBD2*01	SGH.....AT	FQN....NGV	CASS PEGGD	EQFF 1
	KG 27	ClPPBF+ Endo-	TRAV21*02	TRAJ40*01	DSA.....IYN	IQSS.....QRE	CAV	K GTYKYIF	TRBV27*01	TRBJ2-3*01	TRBD1*01 F	MNH.....EY	SMN....VEV	CASSL GAN	TDTQYF 1
	4K	ClPPBF+ Endo-	TRAV22*01	TRAJ27*01	DSV.....NN	IPS.....GT	CA	DRS AGKSTF	TRBV6-2or -3	TRBJ2-1*01	TRBD2*01 F	MNH.....EY	SVG.....EGT	CASSY WGGAIAGLPG	NEQFF 2
	C3	ClPPBF+	TRAV23*01	TRAJ32*01	NTA.....FDY	IRPD.....VSE	CAAS	IL YGGATNKLIF	TRBV6-5*01	TRBJ2-7*01	TRBD2*01	MNH.....EY	SVG.....AGI	CASSY SYRTSG	YEQYF 2
	4K	ClPPBF+ Endo-	TRAV26-1	TRAJ48*01	TISG.....NEY	GLK.....NN	CIVRV	VV FGNEKLTF	TRBV6-1	TRBJ1-2*01	TRBD1*01	MNH.....NS	SAS.....EGT	CASS PGWTGP	NYGYTF 37
	KG 28	ClPPBF+ Endo-	TRAV26-2*01	TRAJ30*01	TISG.....TDY	GLT.....SN	CILRD	GWLG DDKIIF	TRBV7-2*01	TRBJ2-5*01	TRBD2*01 F	SGH.....TA	FQG....NSA	CASS SGTSVRVV	QETQYF 3
	KG 28	ClPPBF+ Endo-	TRAV26-2*01	TRAJ22*01	TISG.....TDY	GLT.....SN	CILRD	PLQ SGSARQLTF	TRBV5-5*01 ,	TRBJ2-3*01	TRBD2*01 F	SGH.....KS	YYE.....KEE	CASSD TPGQGVYFIG	SDTQY 1
	CA4	ClPPBF+ Endo-	TRAV27*01	TRAJ28*01	SVF.....SS	VVTG.....GEV	CAG	RVTP GAGSYQLTF	TRBV2*01	TRBJ2-2*01	TRBD2*02	SNH.....LY	FYN.....NEI	CAS RILAGA	TGELFF 1
T27_B7	CA4	ClPPBF+ Endo-	TRAV27*01	TRAJ56*01	SVF.....SS	VVTG.....GEV	CAG	YN GANSKLTF	TRBV27*01	TRBJ2-2*01	TRBD2*01	MNH.....EY	SMN....VEV	CASS FISSY	TGELFF 11
	4D	ClPPBF+ Endo-	TRAV29*01	TRAJ49*01	NSM.....FDY	ISSI.....KDK	CAAS	VSH TGNQFYF	TRBV4-2*01	TRBJ1-5*01	TRBD2*01	LGH.....NA	YNF.....KEQ	CASSQ DWVT	NQPQHF 1
A29B14	KG29	ClPPBF+ Endo-	TRAV29*01	TRAJ34*01 F	NSM.....FDY	ISSI.....KDK	CAA	TFIH NTDKLIF	TRBV14*01 F	TRBJ1-1*01	TRBD2*02 F	SGH.....DN	FVK.....ESK	CASSQ DRVG	EAFF 2
T29_E	CA3	ClPPBF+ Endo-	TRAV29*01	TRAJ16*01	NSM.....FDY	ISSI.....KDK	CAAS	EP SDGQKLLF	TRBV19*01	TRBJ2-7*01	TRBD1*01	LNH.....DA	SQI.....VND	CAS RDS	SYEQYF 12
	CA4	ClPPBF+ Endo-	TRAV29*01	TRAJ37*01	NSM.....FDY	ISSI.....KDK	CAA	FLPLH SGNTGKLIF	TRBV4-2*01	TRBJ1-4*01	TRBD2*01	LGH.....NA	YNF.....KEQ	CASSQ DWGG	NEKLFF 1
	KG 28	ClPPBF+ Endo-	TRAV3*01	TRAJ6*01	VSG.....NPY	YITG.....DNLV	CAV	ET GGSYPTF	TRBV25-1*01	TRBJ2-1*01	TRBD2*02 F	MGH.....DK	SYG.....VNS	CASSE LYEGG	SYNEQFF 1
	CA4	ClPPBF+ Endo-	TRAV34*01	TRAJ31*01	KTL.....YG	LQKG.....GEE	CGAD	GG NNNARLMF	TRBV5-4*01	TRBJ1-1*01	TRBD1*01	SGH.....NT	YYR.....EEE	CASSL LGM	AFF 1
	CA4	ClPPBF+ Endo-	TRAV35*01	TRAJ34*01	SIF.....NT	LYKA.....GEL	CA	ST SYNTDKLIF	TRBV28*01	TRBJ2-5*01	TRBD1*01	MDH.....EN	SYD....VKM	CASS DLTGQG	ETQYF 1
	CH8	ClPPBF+ Endo-	TRAV38-2*01	TRAJ21*01	TSES.....DYY	QEAY...KQQN	CA	LL YNFNKFYF	TRBV11-2*03	TRBJ2-4*01	TRBD1*01	SGH.....AT	FQN....NGV	CASSL VAGPL	AKNIQYF 7
	KG 28	ClPPBF+ Endo-	TRAV1-2*01	TRAJ10*01	TSG.....FNG	NVL.....DGL	CAV	SG ILTGGGNKLTF	TRBV13*01	TRBJ2-3*01	TRBD2*02 F	PRH.....DT	FYE.....KMQ	CAS SPALEREG	TDTQYF 1
	KG 27	ClPPBF+ Endo-	TRAV10*01	TRAJ3*01	VSP.....FSN	MTFS.....ENT	CVV	AG SSASKIIF	TRBV20	TRBJ1-1*01	TRBD1*01 F	DFQ.....ATT	SNEG..SKA	CSAR EVGV	EAFF 1
	KG 28	ClPPBF+ Endo-	TRAV4*01	TRAJ29*01	NIAT.....NDY	GYK.....TK	CLVGD	GIWQ GNTPLVF	TRBV2*01	TRBJ2-3*01	TRBD2*01 F	SNH.....LY	FYN.....NEI	CASS LRP	DTQYF 2
	KG 27	ClPPBF+ Endo-	TRAV5*01	TRAJ44*01	DSS.....STY	IFSN.....MDM	CA	QL TGTASKLTF	TRBV11-2*01	TRBJ2-7*01	TRBD1*01	SGH.....AT	FQN....NGV	CASS FDPV	YEQYF 1
	CH8	ClPPBF+ Endo-	TRAV8-1*01	TRAJ34*01	YGG.....TVN	YFSG...DPLV	CAV	IH YNTDKLIF	TRBV11-3	TRBJ1-1*01	TRBD2*01	SGH.....NT	YEN.....EEA	CASSL GGLD	TEAFF 1
	KG 29	ClPPBF+ Endo-	TRAV8-2*01	TRAJ45*01	SSY.....SPS	YTSA...ATLV	CVVS	DHS GGGADGLTF	TRBV2*01	TRBJ2-5*01	TRBD2*01 F	SNH.....LY	FYN.....NEI	CAS RPGLE	ETQYF 3
	CA2	ClPPBF+ Endo-	TRAV9-2*01	TRAJ28*01	ATG.....YPS	ATKA.....DDK	CA	PP GAGSYQLTF	TRBV24-1*01	TRBJ2-6*01	TRBD1*01	KGH.....DR	SFD.....VKD	CATSDL RR	SGANVLTF 1

			TCRδ						TCRγ					
			TRDV	TRDJ	TRDD	CDR1δ	CDR2δ	CDR3δ	TRGV	TRGJ	CDR1γ	CDR2γ	CDR3γ	
VD1G9	C3	ClPPBF+	TRDV1*01	TRDJ1*01	TRDD2*01	TSWW.....SYY	QG.....S	CALGE KHIFPNPWGTT KLIF	TRGV9*01	TRGJ1*02	GITI...SATS	ISYD.....GTV	CALWEV YYKKLF	20
	2N	ClPPBF+	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE RVFLILGN TDKLIF	TRGV5*01	TRGJ1*02	VIN.....AFY	YDVS...NSKD	CATWD PRG KLF	11
	2N	ClPPBF+	TRDV1*01	TRDJ1*01	TRDD1*01 or 3*01	TSWW.....SYY	QG.....S	CALGE LDKSLGDPY TDKLIF	TRGV9*01	TRGJ1*01	GITI...SATS	ISYD.....GTV	CALWE ASPTLVN YYKKLF	3
	4d	ClPPBF+	TRDV1*01	TRDJ3*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE FRWLPTGFS SWDTRQMFF	TRGV5*01	TRGJ1*02	VIN.....AFY	YDVS..NSKD	CATWDR R YYKKLF	1
	4d	ClPPBF+	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE LDKSLGDPY TDKLIF	TRGV2*02	TRGJ1*01	EGS.....NGY	YDSY..NSKV	CATWDG RG YKKLF	1
VD1G4	CA1	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE ALLPGGYEY TDKLIF	TRGV4*01	TRGJ1*02	EGS.....TGY	YDSY..TSSV	CATWDG RVH YKKLF	6
	CA3	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE HAFPPGFPDRY TDKLIF	TRGV5*01	TRGJ1*02	VIN.....AFY	YDVS..NSKD	CATWD GN KKLF	3
	CH7	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*01	TSWW.....SYY	QG.....S	CALG DHTLPVTGGFLR TDKLIF	TRGV5*01	TRGJ1*02	VIN.....AFY	YDVS..NSKD	CATWDR REN KKLF	5
	CH7	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE LTLPPGGYAA DKLIF	TRGV5*01	TRGJ1*02	VIN.....AFY	YDVS..NSKD	CATWDR YKKLF	5
	CH7	ClPPBF+ Endo-	TRDV1*01	TRDJ3*01	TRDD3*01	TSWW.....SYY	QG.....S	CALG VPTGGYA QMFF	TRGV2*02	TRGJ1*02	EGS.....NGY	YDSY..NSKV	CAT RHG YYKKLF	7
	KG 28	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD3*01	TSWW.....SYY	QG.....S	CALGE LSVRGRH TDKLIF	TRGV5*01	TRGJP2*01	VIN.....AFY	YDVS..NSKD	CATWD VRSSD WIKTF	1
	KG 28	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE PHLPGAIHPKY TDKLIF	TRGV8*01	TRGJ2*01	VEN.....AVY	YDSY..NSRV	CATWD YYKKLF	13
	KG 28	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALGE RFLWYWGSD KLIF	TRGV5*01	TRGJP2*01	VIN.....AFY	YDVS..NSKD	CATWDR SSSD WIKTF	3
	KG 28	ClPPBF+ Endo-	TRDV1*01	TRDJ1*01	TRDD2*01 or 3*02	TSWW.....SYY	QG.....S	CALG PLSALPSYWGRY TDKLIF	TRGV3*01	TRGJ1*02	VTN.....TFY	YDVS..TARD	CATWD QN YYKELF	2
	2N	ClPPBF+	TRDV2*03	TRDJ1*01	TRDD3*01	GEAI....GNYY	EK.....D	CACD YVLGET TDKLIF	TRGV9*01	TRGJP*01	GITI...SATS	ISYD.....GTV	CALWEV QW ELGKKIKVF	3
	4d	ClPPBF+	TRDV2*03	TRDJ1*01	TRDD3*01	GEAI....GNYY	EK.....D	CACD KVGAAY TDKLIF	TRGV9*01	TRGJP*01	GITI...SATS	ISYD.....GTV	CALWEV QELGKKIKVF	2

IMGT TCR gene nomenclatures and associated complementary determining region (CDR) loop amino acid sequences are shown or αβ (A) and γδ (B) T cells sorted with CD1d-ClPPBF tetramers post-tetramer–associated magnetic enrichment—Fig. 3*A* (donor C3)—or as CD1d-ClPPBF tetramer$^+$CD1d-endo tetramer—after CD1d-ClPPBF tetramer sort/expansion in culture for 21 d as per Fig. 3*C* (*Upper Left* quadrants). The number of observations (obs. column) refers to the frequency at which each unique clonotype was observed from an analysis of 13 donors (αβTCRs) or 7 donors (γδTCRs). A total of six sorting experiments were performed. Red residues are either partially or fully nongermline encoded. Residues in bold highlight GG patterns in CDR3-α/δ loops. XX means undetermined. Sequences highlighted in blue and orange correspond to sequences selected to generate αβ and γδ TCR-transduced NKT cell clones, respectively. Amino acid positions: CDR1-IMGT (27 to 38), CDR2-IMGT (56 to 65), and CDR3 (105 to 117).

(Val72 and Gln150). No other mutations impacted on the activation of the VD1G9 cell line, which may suggest that this TCR engages CD1d residues that were not mutated and/or that the loss of individual contact residues was insufficient to reduce signaling. A12B30 was affected by mutations in residues that spanned from the extreme A′ side (Gln62) to the central region of the antigen portal (Val72 and Glu83), with His68-Ala and Arg79-Ala mutations enhancing activation in the absence of ClPPBF.

Overall, the CD1d docking footprints of the PPBF-reactive TCRs were distinct from the F′ docking of type I NKT TCRs (Fig. 4) (40, 41), α-GlcADAG, or α-GalCer–reactive TRAV13–TRAJ50$^+$ TCRs (37, 42). These footprints were also distinct to those described for other type II and atypical NKT TCRs (34, 38,39, 43, 44). Our results suggest that PPBF-reactive TCRs commonly interact with Trp160 of CD1d and dock over the central antigen-binding cleft. This may allow productive analysis of molecules that are trapped between CD1d and TCR using TCR trap technology (24, 25).

Trapping Lipids in CD1d-PPBF-TCR Complexes. Since PBFs promote CD1d-TCR binding (Fig. 3 *C* and *E*), this raises the question of whether PBFs either alter the structure of CD1d, influence lipid ligand content, or are captured at the CD1d-TCR interface. To address this, we generated soluble ABd TCR to trap and identify compounds within CD1d-TCR complexes. The TCR trap approach

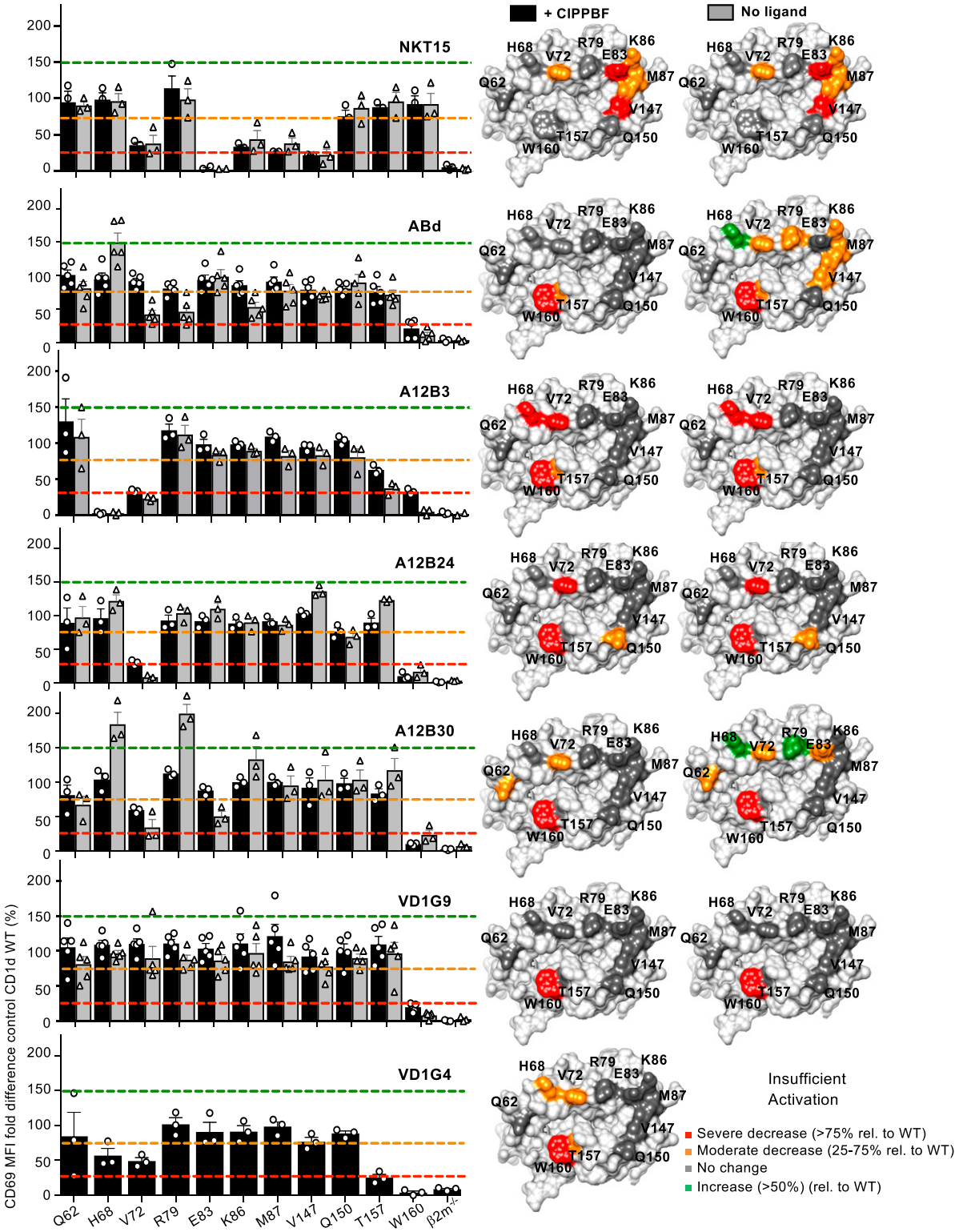

Fig. 4. CD1d docking modes of PPBF-reactive type II NKT cells. ABd, A12B3, A12B24, A12B30, VD1G9, and VD1G4 PPBF-reactive NKT TCR-transduced SKW3.β2m$^{-/-}$ cell lines were cocultured for 16 h with C1R cells each transduced with a single (Ala) mutated version of CD1d, in the presence of ClPPBF at 10 μM (black) or without ligand (gray). The responses by the type I NKT15 TCR-expressing cell line was also analyzed as control. The level of activation (CD69-fold increase in comparison to TCR-transduced cell lines alone) elicited by each mutant line is normalized to the response elicited to WT C1R.CD1d shown in *SI Appendix*, Fig. S4C. Data are from $n = 3$ independent experiments + SEM, except for ABd and VD1G9 in which $n = 5$. Corresponding CD1d surface maps (Protein Data Bank code: 1Zt4) are shown to the right of each graph, depicting residues that when mutated had no effect (dark gray).

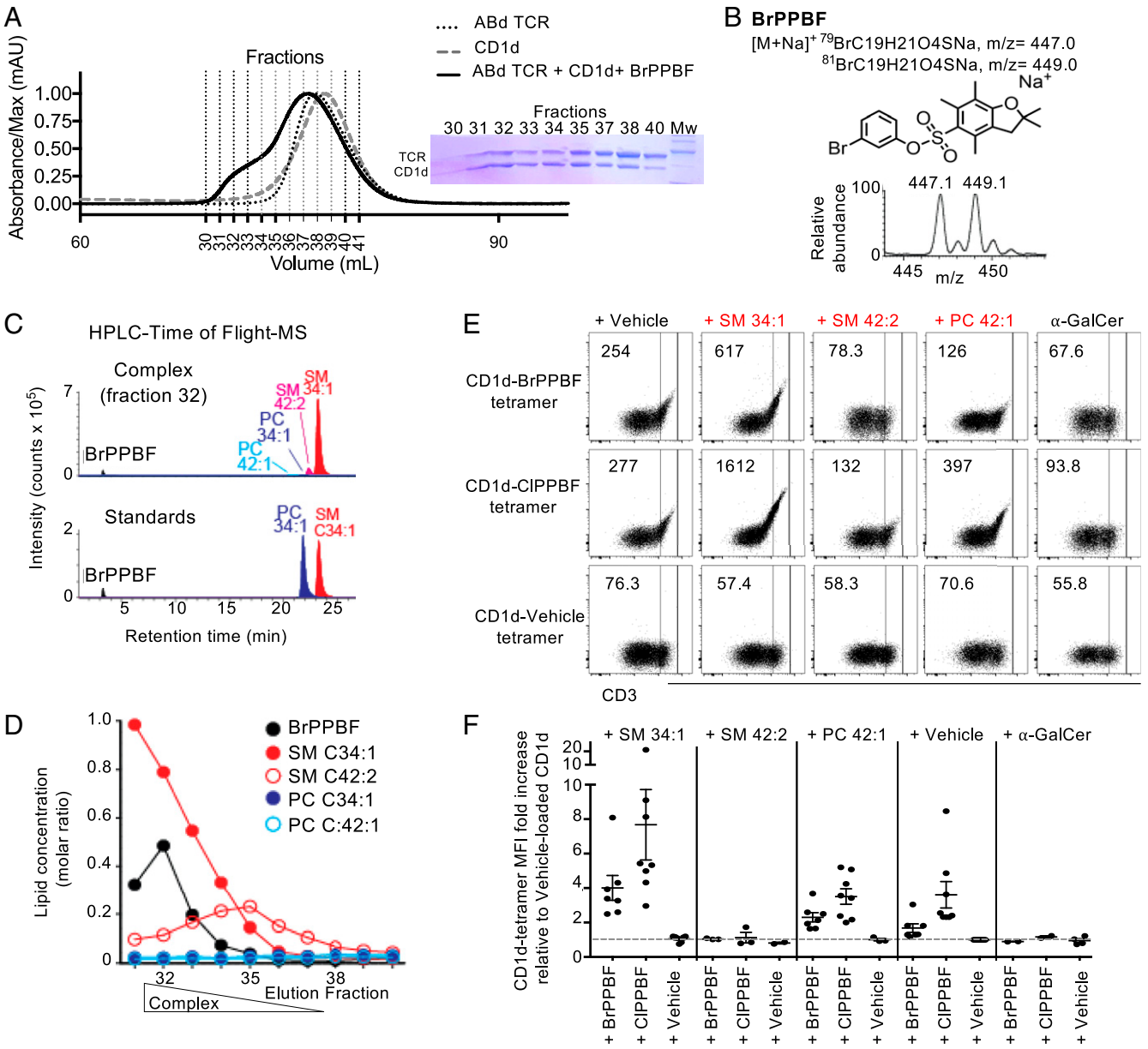

Fig. 5. TCR trap determination of CD1d-BrPPBF-TCR complexes. (*A*) CD1d-TCR complexes were separated from uncomplexed CD1d and TCR by size using fast-performance liquid chromatography and protein composition and validated by polyacrylamide gel electrophoresis. Fractions were normalized to protein input and then subjected to lipid elution and analyzed by mass spectrometry (MS). (*B*) BrPPBF was identified by shotgun nano-electrospray ionization MS in the early complex (fraction 32). (*C*) Lipids extracted from the complex (fraction 32) were analyzed by HPLC–Quadruple Time of Flight –MS to obtain the accurate mass and retention time in comparison with the indicated standards. (*D*) The relative quantities of five detected lipids in the TCR trap fractions were estimated by fitting the chromatogram area to an external standard curve. (*E*) The ABd TCR$^+$ cell line was stained with CD1d-ClPPBF or BrPPBF preloaded with short chain 34:1 SM or long chain 42:2 SM or 42:1 PC. Representative flow cytometry plots and MFI of CD1d tetramer staining of cells with similar TCR levels (*Top*). The fold increase in CD1d tetramer MFI is shown with each combination of lipid and PBF (*Bottom*). Each point represents one individual experiment.

(24, 25) involves mixing purified recombinant CD1d proteins and TCRs followed by size exclusion chromatography to enrich for CD1d-TCR complexes, which are larger and thus elute earlier than CD1d and TCR alone (Fig. 5*A*). Mass-spectrometric (MS) analysis of lipid eluents derived from early eluting protein (fractions 30 to 34) allows direct identification of antigen molecules within CD1d-TCR complexes, which are compared to ligands present within intermediate (fractions 35 to 36) and later fractions that coelute with protein monomers (fractions 37 to 41) (24, 25). The addition of BrPPBF to mixtures of CD1d and the ABd TCR facilitated CD1d-TCR complex formation as observed by the early elution of material (fractions 30 to 34) compared to the profiles of CD1d alone or TCR alone (Fig. 5*A*). This supports that BrPPBF increased formation of CD1d-TCR complexes.

Nano-Electrospray Ionization of Lipids in CD1d-TCR Complexes. Sensitive shotgun nano-electrospray MS analysis of the trace lipids eluted from proteins generated an overview of the compounds present in TCR-CD1d complexes (fraction 32), intermediate (fraction 35), and noncomplexed (fraction 38) fractions (*SI Appendix*, Fig. S5 *A* and *B*). Diagnostic ions corresponding to the sodium adduct of BrPPBF (*m/z* 447.1 and 449.1) and matching the 1:1 isotopic ratio of ^{79}Br and ^{81}Br were detected in fraction 32 (Fig. 5*B*), demonstrating the presence of BrPPBF within the CD1d-TCR complexed fraction.

CD1d-endo complexes also carried endogenous lipids acquired during CD1d synthesis in the mammalian expression system used, which were identified in all fractions (Fig. 5*C* and *SI Appendix*, Fig. S5*B*). Collision-induced dissociation (CID) MS of the CD1d-TCR complex fraction revealed a prominent ion with *m/z* 725.4, identified as short-chain sphingomyelin (SM) with a combined chain length of C34 with one unsaturation (34:1 SM) (*SI Appendix*, Fig. S5 *B* and *D*). We also detected weak signals for two phosphatidylcholines, 34:1 PC and 42:1 PC (*m/z* 782.4 and 894.6) (*SI Appendix*, Fig. S5 *B* and *C*) and 40:2 SM and 42:2 SM (*m/z* 807.5 and 835.5) (*SI Appendix*, Fig. S5*B*). While 34:1 SM was enriched within CD1d-TCR fractions, the other ions were enriched in uncomplexed fractions (*SI Appendix*, Fig. S5*B*), indicating BrPPBF is within the CD1d-TCR complex and influences the spectrum of endogenous lipids found within the TCR-CD1d complex.

Quantitative Analysis of Trapped Lipids by High-Performance Liquid Chromatography–MS. We used high-performance liquid chromatography (HPLC)-MS, which provides higher mass accuracy and uses chromatography to assess the retention time of eluted ligands versus standards. This approach confirmed the identity of BrPPBF and 34:1 SM in the CD1d-TCR complex fractions as well as trace amounts of 42:2 SM, 34:1 PC, and 42:1 PC (Fig. 5*C*). Time-of-flight (TOF) HPLC-MS detection allows quantitation of these molecules using external standards (Fig. 5*D* and *SI Appendix*, Fig. S6). These measurements demonstrated a clear pattern for selective capture of 34:1 SM and BrPPBF in early complex fractions. 42:2 SM and PCs were present only at low levels and showed modest variations but less clear enrichment patterns than seen with 34:1 SM (Fig. 5*D*). Importantly, control runs of CD1d alone indicated that these patterns of lipid or BrPPBF enrichment in early fractions did not occur in the absence of TCR (*SI Appendix*, Fig. S7), so fractionation was a result from lipid/BrPPBF entrapment by the TCR rather than direct interaction with the column.

To investigate whether BrPPBF was required for CD1d-TCR interaction, we repeated the TCR trap experiment in the absence of BrPPBF. Lower levels of complexation were observed, with much less material obtained in the earliest fractions (*SI Appendix*, Fig. S7). Both 34:1 SM and 42:1 SM were detected in early CD1d-TCR complex fractions, but the ratio of short (34:1) to long (42:2) SM was markedly increased in the early fractions in the presence of BrPPBF (*SI Appendix*, Fig. S5*B*). Overall, these experiments indicated that BrPPBF promotes early elution of CD1d-TCR complexes but that short-chain SM can also appear in CD1d-TCR complexes. Finally, we studied whether 34:1 SM augments CD1d tetramer staining when added as an exogenous ligand. For CD1d-endo tetramers, exogenous 34:1 SM alone did not enhance staining of the ABd TCR (Fig. 5 *E* and *F*) or the VD1G9 TCR (*SI Appendix*, Fig. S8). However, when CD1d tetramers were coloaded with BrPPBF or ClPPBF and 34:1 SM, staining augmentation was observed, suggesting synergistic interaction of BrPPBF with a self-lipid. Conversely, coloading of BrPPBF or ClPPBF with 42:2 SM led to inhibition of CD1d tetramer staining when compared to coloading with vehicle, while coloading 34:1 PC had no impact. These results suggest that the ability of PBF family molecules to enhance CD1d-TCR interactions is lipid sensitive.

Discussion

While most studies of CD1 emphasize T cell recognition of lipids comprised of alkyl chains, this study provides a different view of NKT cell activation by constrained, nonlipidic small molecules in the phenyl sulfonate family. Furthermore, this work broadens our knowledge of the diversity of the type II NKT cell family by defining a public TCR motif among polyclonal T cells and by providing detailed information about the identity and action of nonlipidic NKT cell agonists. Whereas initial views favored an indirect or pharmacological action of these atypical molecules, we identified and characterized a polyclonal population of NKT cells that can recognize, via their TCR, CD1d molecules associated with an exogenously loaded nonlipidic PBF molecule and an endogenous self-lipid, such as 34:1 SM. Overall, polyclonal αβ and γδ T cells reactive to CD1d and PBF family molecules were present among type II NKT cells in all donors tested.

Notably, one of the biased TCR motifs (TRAV12–TRAJ6) that emerged among type II NKT cells resembled the TCR from the original ABd clone isolated 17 y prior to this study (15). This finding and the monomorphic nature of CD1d hint at conserved modes of interaction of the TCR-PBF-CD1d interaction. The ABd TCR and polyclonal TCRs stained by CD1d-ClPPBF tetramers showed tolerance to some, but not all, variations in the PBF molecular structure, suggesting that the 3-substituted phenyloxysulfonyl motif is critical for supporting the TCR-CD1d interaction, while the nature of the benzofuran/chroman system is more flexible.

Models of glycolipid display by CD1d generally predict that the two alkyl chains present in most glycolipid antigens occupy the two pockets of CD1d, and the carbohydrate head group protrudes for TCR recognition (45). However, PPBF variants are small (*m/z* 350 to 400) and rigid molecules constrained by one phenyl and one sulfonyl group; these nonlipidic molecules lack alkyl chains and head groups and therefore do not neatly fit within the amphipathic lipid-display model paradigm. Although the precise mode of lipid and small molecule positioning on CD1d awaits further structural confirmation, our data provide specific insights into candidate mechanisms. Firstly, our data demonstrate that PBFs are not mitogens that directly or broadly activate T cells. Instead, CD1d tetramers and TCR^+ cell line assays indicate that PBFs are directly involved in promoting CD1d-TCR interactions. However, some T cells identified using CD1d tetramers loaded with ClPPBF did not absolutely require PBF for tetramer staining or CD1d-TCR complex formation, suggesting that these clones have a basal reactivity toward CD1d presenting endogenous ligands, which in some cases can be enhanced in the presence of PBFs. Intriguingly, these findings are extended by the unbiased discovery of molecules eluted from a CD1d-αβTCR complex, which detected BrPPBF and a self-lipid 34:1 SM in eluates. This finding shows that PBF both increases CD1d-TCR complex formation and places a PBF within the CD1d-TCR complex, raising the question of whether PBF and 34:1 SM can jointly or separately support TCR binding. The TCR trap assay without BrPPBF also revealed an enrichment of a short-chain SM in early eluting fractions in which CD1d-TCR complexes are likely to emerge, suggesting that a self-lipid can support CD1d-TCR binding at a threshold that does not detectably activate or enable CD1d tetramer staining of NKT cells such as the ABd clone.

The action of PBFs to increase CD1d-mediated activation, tetramer binding, and TCR trapping/complexation are subject to two possible interpretations to address the key question of whether PPBF directly engages the TCR at the binding interface or whether it indirectly acts on CD1d and/or endogenous lipids. One is based on the fact that stimulatory SMs and PBFs are both relatively small molecules. The mass of PBFs (~400 amu) is about half the size of most lipids bound to human CD1d [~800 amu (46)], and the ratio of 34:1 SMs to 42:2 SMs is increased in complexes. Recent studies show how small "permissive" ligands buried within CD1a can expose the CD1a surface for direct recognition (47–52). In this interpretation, the reactivity of ABd and other TCRs might be explained by the small size of short-chain SM and PBF molecules, allowing them to bind largely within the CD1d cleft to expose the outer surface of CD1d to a TCR. Consistent with this interpretation, footprint analysis shows that four αβ and two γδ TCRs bind over the antigen portal of CD1d where larger ligands would protrude. However, this analysis does not explain why SM alone does not lead to T cell staining with CD1d tetramers, whereas ClPPBF or BrPPBF and SM (C34:1) synergize to enhance the TCR-CD1d interaction. The other interpretation

is a combined action consistent with the TCR trap experiments showing that both SM and BrPPBF were in CD1d-TCR complexes, although this does not necessarily mean that the two molecules are present together within the same complexes. However, it is possible that the ABd TCR is recognizing a trimolecular complex comprising CD1d, SM (C34:1), and BrPPBF which is also consistent with the combination of these two ligands enhancing CD1d tetramer staining of PPBF-reactive cell lines. In this model, the TCR may directly contact both SM or Cl/BrPPBF, or SM may alter the orientation of Cl/BrPPBF, or vice versa, in order to create a better TCR epitope with one or the other.

The modulation of type I NKT cell responses to CD1d-lipid antigen complexes through lipid tail (53) or headgroup modifications (54–56) has been extensively studied and can impact on immune response and disease outcomes (57). Our results show that a family of structurally related nonlipidic small molecules can also modulate CD1d-dependent T cell responses and could be used as drugs to transiently modulate CD1d-mediated T cell responses for immunotherapy. Finally, PBF compounds share chemical features with commonly used sulfa drugs that generate hypersensitivity reactions in humans, including sulfisomidine, sulfadiazine, sulfasalazine, and celecoxib (16). In a similar vein, small lipid allergens can enhance CD1a-mediated T cell autoreactivity by displacing certain lipids from CD1a and altering the remaining lipid repertoire, enhancing CD1a autoreactivity by some T cell clones (50). Given that hypersensitivity to sulfa drugs limits the use of these otherwise valuable therapeutics (18, 19), these data support the investigation of whether PBF-like sulfa drugs can bind CD1 proteins and whether there is a role for type II NKT cells in sulfa drug–induced hypersensitivity.

Materials and Methods

PBMCs were derived from blood donors and prepared as previously described (37). The PBF analogs were synthesized in house (*SI Appendix, SI Chemistry Experimental*), and lipids were purchased commercially. Human CD1d was produced in house from mammalian cells similar to that previously described (58). The TCR trap assay was performed as previously described with minor changes (24, 25). Mass spectrometry analyses were performed on a Thermo Fisher LXQ Ion Trap mass spectrometer or an Agilent 6520 Accurate-Mass Q-TOF with HPLC. For details of experimental conditions and analysis, see *SI Appendix, SI Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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