Photoaffinity Antigens for Human γδ T Cells[1]

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Vγ2Vδ2 T cells comprise the major subset of peripheral blood γδ T cells in humans and expand during infections by recognizing small nonpeptide prenyl pyrophosphates. These molecules include (*E*)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP), a microbial isoprenoid intermediate, and isopentenyl pyrophosphate, an endogenous isoprenoid intermediate. Recognition of these nonpeptide Ags is mediated by the Vγ2Vδ2 T cell Ag receptor. Several findings suggest that prenyl pyrophosphates are presented by an Ag-presenting molecule: contact between T cells and APC is required, the Ags do not bind the Vγ2Vδ2 TCR directly, and Ag recognition is abrogated by TCR mutations in CDRs distant from the putative Ag recognition site. Identification of the putative Ag-presenting molecule, however, has been hindered by the inability to achieve stable association of nonpeptide prenyl pyrophosphate Ags with the presenting molecule. In this study, we show that photoaffinity analogues of HMBPP, *meta*/*para*-benzophenone-(methylene)-prenyl pyrophosphates (*m*/*p*-BZ-(C)-C_5-OPP), can crosslink to the surface of tumor cell lines and be presented as Ags to γδ T cells. Mutant tumor cell lines lacking MHC class I, MHC class II, β_2-microglobulin, and CD1, as well as tumor cell lines from a variety of tissues and individuals, will all crosslink to and present *m*-BZ-C_5-OPP. Finally, pulsing of BZ-(C)-C_5-OPP is inhibited by isopentenyl pyrophosphate and an inactive analog, suggesting that they bind to the same molecule. Taken together, these results suggest that nonpeptide Ags are presented by a novel-Ag-presenting molecule that is widely distributed and nonpolymorphic, but not classical MHC class I, MHC class II, or CD1. ***The Journal of Immunology,*** **2008, 181: 7738–7750.**

The γδ T cell subset, which expresses T cell Ag receptors (TCR) using γ and δ rearranging genes (1), has functional roles in immunity distinct from the αβ T cell subset (2). In humans, the majority of circulating γδ T cells express Vγ2Vδ2 (also termed Vγ9Vδ2) TCRs. Vγ2Vδ2 T cells recognize nonpeptide prenyl pyrophosphate intermediates in isoprenoid biosynthesis such as (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)[5] (3, 4) and isopentenyl pyrophosphate (IPP) (5). Vγ2Vδ2 T cells can expand during infections to very high numbers, accounting for half of the circulating T cells in some patients (reviewed in Ref. 2). HMBPP is the most potent Ag described (3) and is produced in the methyl-erythritol phosphate pathway for isoprenoid synthesis used by many eubacteria, some protozoa, and plant chloroplasts. By recognizing HMBPP produced by many pathogenic bacteria (such as those that cause tuberculosis and gastroenteritis) as well as apicomplexan parasites (such as those that cause malaria and toxoplasmosis), Vγ2Vδ2 T cells likely play important roles in human immunity to both bacteria and parasites (2).

Vγ2Vδ2 T cells also kill many types of tumor cells in vitro, including malignant B cells, melanomas, prostate carcinomas, renal cell carcinoma, epithelial carcinomas, and others (6–10). This appears due to both TCR-mediated and NK receptor-mediated tumor cell recognition (11–13). Zoledronate and other bisphosphonates greatly enhance tumor recognition by inhibiting the intracellular farnesyl pyrophosphate synthase enzyme, resulting in increases in endogenous IPP (H. Wang and C. T. Morita, unpublished observations and Refs. 14–16). Importantly, treatment of patients with B cell malignancies (17) and metastatic prostate carcinomas (18) with a bisphosphonate and IL-2 to activate and maintain Vγ2Vδ2 T cells led to partial remissions and stable disease in several individuals. Given their broad tumor reactivity, immunotherapy with Vγ2Vδ2 T cells appears to have promise for the treatment of a variety of cancers.

Despite the importance of Vγ2Vδ2 T cells in human immunity to pathogens and their potential for tumor immunotherapy, little is known about the molecular mechanisms for the presentation of prenyl pyrophosphate Ags to these T cells. Although gene transfer

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[5] Abbreviations used in this paper: HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; β_2M, β_2-microglobulin; BZ, benzophenone; BrHPCP, bromohydrin pyrophosphonate; BrHPP, bromohydrin pyrophosphate; DATFP, diazo-3,3,3-trifluoropropionyloxy; EPP, ethyl pyrophosphate; FPP, farnesyl pyrophosphate; FPPS, FPP synthase; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; *m*, *meta*; OPP, pyrophosphate; *p*, *para*.

studies show that the Vγ2Vδ2 TCR mediates Ag recognition by Vγ2Vδ2 T cells (11), there is no evidence for direct binding of prenyl pyrophosphates to the Vγ2Vδ2 TCR. Attempts to soak prenyl pyrophosphates into crystals of the Vγ2Vδ2 TCR (19) or to demonstrate prenyl pyrophosphate binding to soluble γδ TCR by equilibrium dialysis or microcalorimetry failed (C. T. Morita, unpublished observations). Moreover, although the chemical structural requirements for antigenic activity of prenyl pyrophosphates and other phosphoantigens has been extensively studied (20–23), this knowledge has provided little insight into how the Ags are presented.

Unlike protein Ags, prenyl pyrophosphate Ags do not require Ag uptake, processing, or intracellular loading for presentation (24). Moreover, activation of Vγ2Vδ2 T cells is extremely rapid, with calcium flux observed within 90 s upon exposure to IPP (24) and metabolic acidification within 10 s upon exposure to bromohydrin pyrophosphate (BrHPP) (25). Although rapid, the activation of Vγ2Vδ2 T cells by prenyl pyrophosphates still requires cell-cell contact (24, 26) similar to the contact required by αβ T cells during the recognition of peptide Ags presented by MHC class I and II molecules (27). Most human cells are capable of presenting prenyl pyrophosphates (as assessed by indirect stimulation by a bisphosphonate) except for those deficient in accessory molecule ligands (6, 28, 29). In contrast, murine, rat, and hamster cells do not present prenyl pyrophosphates or bisphosphonates (26, 28, 30).

The requirement for cell-cell contact coupled with the small size of prenyl pyrophosphates (they are likely monovalent) and their lack of direct binding to the Vγ2Vδ2 TCR suggest that prenyl pyrophosphates are presented by a presenting molecule, similar to peptides presented by MHC class I and class II or lipids presented by CD1. However, unlike peptide Ags, prenyl pyrophosphates do not stably associate with their presenting molecule with high affinity, precluding pulsing of these Ags on APC (24, 26). This type of presentation is similar to that of nonpeptide drugs, such as sulfamethoxazole and lidocaine, that load into MHC class I and II molecules on the cell surface for recognition by CD8 and CD4 αβ T cells (31, 32). Although this recognition is MHC restricted, the drugs do not stably associate with MHC molecules or require internalization (31, 33). Recognition of lipid Ags that load into CD1a or CD1b molecules at the cell surface also show similarities to prenyl pyrophosphate recognition, because the lipids bind extremely rapidly (as short as 2 min) and, again, do not require processing or internalization (34, 35). However, the putative presenting molecule for prenyl pyrophosphates has eluded identification.

Previously, we found that prenyl pyrophosphate Ags did not stably associate with APC with high affinity because EBV-transformed B cells and PBMC pulsed with IPP or mono-ethyl-phosphate followed by washing did not activate Vγ2Vδ2 T cells (24). This direct presentation of prenyl pyrophosphates (24) differs from the indirect stimulation by bisphosphonates, which enter cells via fluid phase endocytosis (36) to inhibit farnesyl pyrophosphate synthase (FPPS) and thus "pulse" into APC (37). Our attempts to measure binding of IPP to the APC cell surface suggest that the binding affinity between prenyl pyrophosphates and any putative Ag-presenting molecule is very low (data not shown). This property has made efforts to characterize the Ag-presenting molecule using natural Ags, such as IPP and HMBPP, difficult.

In this study, we sought a prenyl pyrophosphate Ag that would have both antigenic activity for Vγ2Vδ2 T cells and stable association with the APC cell surface. We show that *meta*/*para* (*m*/*p*)-benzophenone (BZ)-(methylene)-prenyl pyrophosphate (*m*/*p*-BZ-(C)-C_5-OPP, where OPP is pyrophosphate), photoaffinity FPP analogues, are recognized by Vγ2Vδ2 T cells. *m*/*p*-BZ-(C)-C_5-OPP stimulate Vγ2Vδ2 T cells even after UV crosslinking to the APC surface and extensive washing. This covalent surface crosslinking is inhibited by IPP, suggesting that the molecules bind to the same protein on the APC cell surface. We also find that natural prenyl pyrophosphate can rapidly "pulse" onto the surface of APC under optimal conditions. *m*-BZ-C_5-OPP was able to stably associate with the cell surface of most hematopoietic and nonhematopoietic cell lines, including mutant APC lacking classical MHC class I, β_2-microglobulin (β2M), MHC class II, and CD1 molecules. Thus, *m*/*p*-BZ-(C)-C_5-OPP Ags may enable identification of this putative presenting molecule, which is predicted to be broadly distributed, functionally nonpolymorphic, and not a known presenting molecule.

Materials and Methods

Antigens

HMBPP was synthesized as described (38). Mono-ethyl phosphate and mono-ethyl pyrophosphate (EPP) were prepared and purified by anion exchange as described (23, 39). Mono-methyl phosphate, farnesyl pyrophosphate (FPP), and IPP were obtained from Sigma-Aldrich. PHA-P was obtained from Difco.

Synthesis of photoaffinity compounds and bromohydrin pyrophosphonate

Syntheses of *m*/*p*-BZ-C_5-OPP were performed by minor modifications of the previous method (40). Briefly, dimethylallyl alcohol was first protected as the chloroacetate and then oxidized with *t*-butyl hydroperoxide and catalytic H_2SeO_3. The resulting aldehyde was reduced with sodium borohydride, and the corresponding allylic alcohols were coupled under Mitsunobu conditions with either 4-benzoylphenol or 3-benzoylphenol to give the protected prenyl benzophenone ethers. The chloroacetate was removed by hydrolysis with methanolic aqueous ammonia, and the allylic alcohols were converted to the corresponding allylic chlorides using *N*-chlorosuccinimide and dimethyl sulfide in dichloromethane. Displacement of the allylic chlorides with tris(tetra-*n*-butylammonium) hydrogen diphosphate afforded the desired allylic diphosphates, which were then purified by reversed-phase chromatography and characterized by nuclear magnetic resonance. Syntheses of *m*/*p*-BZ-C-C_5-OPP (ether) were performed as described (41). Syntheses of *m*/*p*-BZ-C-C_5-OPP (esters) were performed as described (42). Syntheses of *m*/*p*-BZ-C-geranyl pyrophosphate (GPP) (ethers) were performed as described (43). Syntheses of 2-diazo-3,3,3-trifluoropropionyloxy (DATFP)-dihydroester-(alkyl)-FPP, DATFP-dh-GPP, and FPP-*p*-BZ were performed as described (M. L. Hovlid, R. L. Edelstein, F. Lopez-Gallego, S. A. Agger, C. Schmidt-Dannert, S. Sen, D. Shintani, K. Cornish, and M. D. Distefano, manuscript in preparation).

Synthesis of bromohydrin pyrophosphonate (BrHPCP; [[(4-bromo-3-hydroxy-3-methylbutoxy)hydroxyphosphinyl]methyl]-phosphonic acid (triammonium salt)) was performed as follows. To a solution of *O*-isopentenyl methylene-1,1-bisphosphonate triammonium salt (5 mg) in water (1 ml) was added freshly prepared bromine water, dropwise, until the solution was persistently yellow. The yellow color (due to a trace amount of Br_2) was removed by gently blowing N_2 into the solution, which was then used without further purification.

Maintenance of cell lines

Va2 cells are derived from the SV40-transformed human fibroblast cell line W1-18 (44–46). Other cell lines used were described previously (24) and include the Burkitt's lymphoma, Raji, and its MHC class II negative mutant, RJ-2.2.5 (47); the $CD3^-$ Jurkat T cell line, JRT3-T3.5 (3); the erythroleukemia cell line, K562 (4); the parent EBV line 721 and the 721.221 mutant that lacks surface expression of HLA-A, -B, and -C (48); and the mutant melanoma cell line, FO-1, which is β_2M deficient (49) and lacks detectable assembled class I molecules (50). Va2 cells were cultured in DMEM (Invitrogen) with 10% FCS (Gemini Bio-Products) at 37°C in a 10% CO_2 incubator while the other cells were cultured at 37°C in a 5% CO_2 incubator in P-medium. P-medium is RPMI 1640 supplemented with 20 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 1× MEM nonessential amino acids, 0.5× MEM essential amino acids, 5.5×10^{-2} mM 2-ME (all from Invitrogen), and 10% FCS (Gemini Bio-Products) and adjusted to pH 7.25 with 2 N NaOH.

Derivation of and culture conditions for Vγ2Vδ2 T cell clones

T cell lines and clones were maintained by periodic stimulation with PHA-P. T cells (1–2 × 10^5/well) were cultured in 1 ml of RPMI 1640 supplemented as for P-medium but with the addition of rIL-2 (1–4 nM; Proleukin, Novartis) and 2% human AB serum (Atlanta Biologicals) with irradiated (4000 rad) allogeneic PBMC (2 × 10^5) and an equal mix of irradiated (5000 rad) EBV-transformed B cells (DG.EBV and CP.EBV) (5 × 10^5 total) as feeder cells and PHA-P (1/4000 final dilution) in 24-well plates (Linbro, MP Biomedicals). The derivation of the CD8$\alpha\alpha^+$ 12G12 and DG.SF68 and the CD4$^+$ HF.2 Vγ2Vδ2 T cell clones has been described (39, 51, 52).

Treatment of APC

APC were treated with either mitomycin C (unfixed) or with glutaraldehyde (fixed). For mitomycin C treatment, APC (1–3 × 10^7 cells/ml) in Dulbecco's PBS without calcium or magnesium were incubated with fresh mitomycin C (Sigma-Aldrich) (100 μg/ml) for 1 h at 37°C in a 5% CO_2 incubator, then washed three times in PBS, and resuspended in either PBS or P-medium for further use. For glutaraldehyde fixation, APC were adjusted to 1–3 × 10^7 cells/ml in PBS and reacted with 0.05% glutaraldehyde (EM grade; Sigma-Aldrich) for 15 s at room temperature while vortexing. The reaction was stopped by adding an equal volume of 0.2 M L-lysine (in H_2O at pH 7.4) followed by incubation for 2 min. The fixed cells were then washed three times in PBS and resuspended in either PBS or P-medium for further use.

Pulsing and UV crosslinking of m/p-BZ-(C)-C_5-OPP on APC

Following mitomycin C-treatment, APC were resuspended in ice-cold PBS to a concentration of 1 × 10^7 cells/ml. The cell suspension (200 μl) was added to wells of a 24-well plate. Two hundred microliters of *m/p*-BZ-C_5-OPP Ag was then added to each well and the cells and Ag were incubated with or without 350-nm UV light treatment for 90 min on ice. The cells were transferred to 15-ml conical tubes (BD Falcon, BD Biosciences), washed three times with 10 ml of ice-cold PBS at 4°C, and resuspended in P-medium for use. In some experiments, the cells were washed first in ice-cold PBS and then exposed to UV light whereas in other experiments they were first exposed to UV light and then washed three times with ice-cold PBS. Long wavelength UV light (350 nm) was used to avoid protein damage. For inhibition of photoaffinity Ag binding by IPP or Br-HPCP, APC were incubated with IPP or BrHPCP (an inactive analog of bromohydrin pyrophosphate) in P-medium with serum for 30 min on ice followed by the addition of a suboptimal dose of either *m*-BZ-C_5-OPP or *m*-BZ-C-C_5-OPP ether and exposed to UV light for 90 min. The APC were then washed three times with 4°C PBS and used as APC with Vγ2Vδ2 T cell clones. Alternatively, BrHPCP was incubated with mitomycin C-treated Va2 for 30 min followed by the addition of *m*-BZ-C-C_5-OPP ether and 12G12 T cells.

Pulsing of prenyl pyrophosphate Ags on APC

Mitomycin C-treated or glutaraldehyde-fixed APC were added at 1 × 10^4–1 × 10^5 cells per 100 μl of PBS into wells of 96-well round-bottom plates (Corning) and incubated with Ags at 37°C in a 5% CO_2 incubator for between 5 and 120 min. APC were washed in the plate 5–7 times with PBS either at room temperature or at 4°C and resuspended in 100 μl of P-medium for further assays. A Vγ2Vδ2 T cell clone was then added to the Ag-pulsed APC and proliferation was assessed by adding 1 μCi of [^{3}H]thymidine at 24 h followed by harvesting 16–24 h later. Each pulsed or unpulsed APC group was also cultured with the same T cell clone in the continuous presence of Ags such as mono-ethyl pyrophosphate, IPP, HMBPP, or *m*-BZ-C_5-OPP, or with the mitogen, PHA-P, as positive controls for each APC group. No proliferation was noted in the absence of T cells. Also, there was no stimulation of T cells in wells pulsed with Ag in the absence of APC.

T cell proliferation and cytokine release assays

T cell proliferation assays were performed as described (53). Briefly, T cells were plated in duplicate or triplicate in round-bottom 96-well plates at 5–10 × 10^4 T cells per well with 1 × 10^5 irradiated (7,000 rad) allogeneic PBMC or mitomycin C-treated allogeneic tumor cells as APC. Because the Vγ2Vδ2 T cell response to prenyl pyrophosphate Ags is not MHC restricted (54), allogeneic cells are suitable APC. The cultures were pulsed with 1 μCi of [^{3}H]thymidine (2 Ci/mmol) on day 1 and harvested 16–24 h later using a Tomtec 96-well harvester. The samples were then counted using a Wallac Betaplate scintillation counter. The mean proliferation and SEM of triplicate (or occasionally duplicate) cultures are shown. For cytokine release, culture supernatants were removed after 24 h and TNF-α or IFN-γ levels determined by sandwich ELISA (R&D Systems) on single or duplicate cultures. For statin inhibition experiments, APC were preincubated with mevastatin (Sigma-Aldrich) for 30 min followed by the addition of set amounts of the stimulatory compounds in the continued presence of mevastatin. T cells were then added after 60 min either directly to the APC (for HMBPP and *m*-BZ-C_5-OPP) or after washing the APC and resuspending them in mevastatin containing medium (for risedronate). Similar results are obtained if the prenyl pyrophosphates are pulsed on the APC.

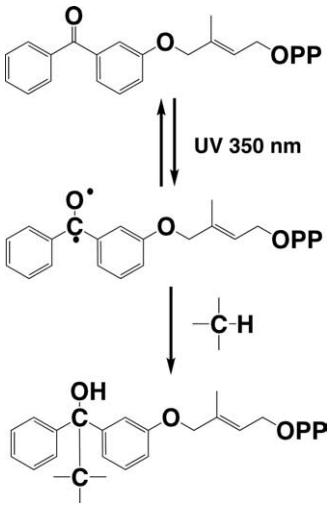

FIGURE 1. Mechanism of cross-linking photoaffinity FPP/HMBPP analogues. *m/p*-BZ-(C)-C_5-OPP compounds (*m*-BZ-C_5-OPP ether is shown) are incubated with the APC and activated by long-wavelength UV light (350 nm; this avoids protein damage), generating a reactive carbon atom. This generation of a reactive carbon intermediate is reversible. When the reactive carbon is present in close proximity to a carbon from the interacting protein (putative Ag-presenting element), it forms a covalent linkage crosslinking the *m/p*-BZ-(C)-C_5-OPP to the interacting protein. Following cross-linking, excess *m/p*-BZ-(C)-C_5-OPP can be washed off, leaving behind covalently attached *m/p*-BZ-(C)-C_5-OPP. This allows *m/p*-BZ-(C)-C_5-OPP to be associated with the APC even after washing.

Results

Benzophenone reaction

Because prenyl pyrophosphate Ags do not stably associate with the putative Ag-presenting molecule, it has been difficult to determine its identity (24). A similar lack of stable association is found for nonpeptide drugs presented as Ags by MHC class I or class II molecules to CD4 and CD8 αβ T cells (31). To overcome this problem, we have studied bioactive photoactivatable analogues of prenyl pyrophosphates to covalently link the prenyl pyrophosphate Ag to the APC surface. The farnesyl pyrophosphate analog *m*-BZ-C_5-OPP is comprised of a BZ photophore linked to an HMBPP molecule via the hydroxyl group (40). *m*-BZ-C_5-OPP is a chemically stable compound that can be reversibly activated using long wavelength (which avoids protein damage) UV light. When activated, *m*-BZ-C_5-OPP reacts with C-H bonds in close proximity (Fig. 1).

m/p-BZ-(C)-C_5-OPP compounds stimulate Vγ2Vδ2 T cells

In in vitro experiments, *m/p*-BZ-C_5-OPP function as analogues of isoprenoid pyrophosphates because they can crosslink to FPPS and other prenyl synthases (40). Structurally, *m/p* -BZ-C_5-OPP resemble both FPP (through their spacing of C-C double bonds) and HMBPP (where the hydroxyl attached to C4 is now an ether or ester bond) (see Figs. 2 and 3 for structures). Therefore, to determine whether *m/p*-BZ-(C)-C_5-OPP compounds are recognized by

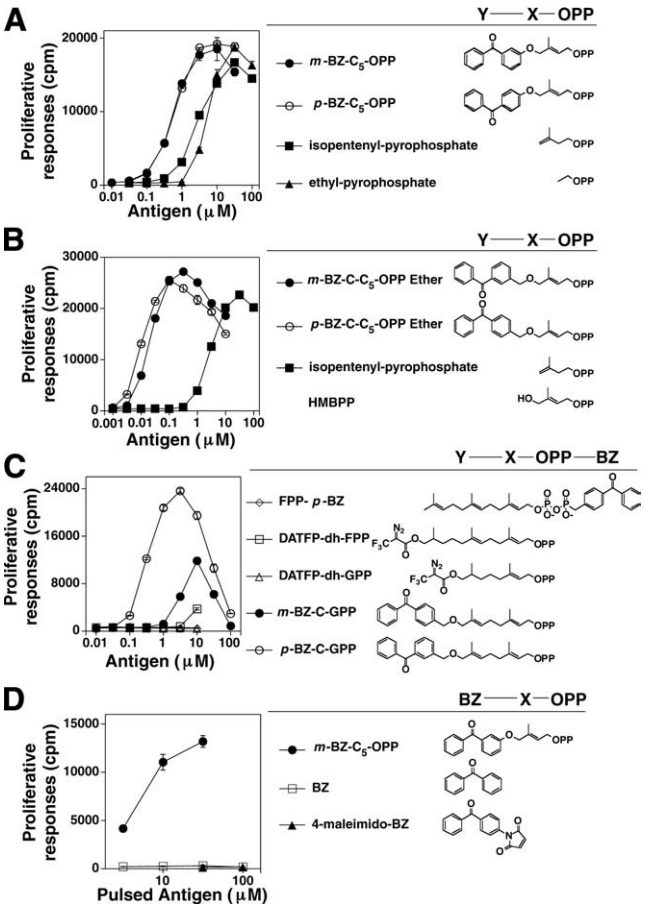

FIGURE 2. Photoaffinity analogues of FPP/HMBPP are Ags for Vγ2Vδ2 T cells. Phosphorylated compounds were incubated with irradiated PBMC and the CD8αα$^+$ 12G12 or DG.SF68 Vγ2Vδ2 T cell clones for 2 days and proliferation was assessed by [^{3}H]thymidine incorporation. Structures of the compounds are shown on the right. "X" refers to the carbon chain closest to the pyrophosphate moiety. "Y" refers to groups spaced away from the pyrophosphate by one isoprenoid unit. *A*, *m*/*p*-BZ-C_5-OPP can activate Vγ2Vδ2 T cells. *m*-BZ-C_5-OPP ether (●), *p*-BZ-C_5-OPP ether (○), IPP (■), and EPP (▲) were incubated with the DG.SF68 Vγ2Vδ2 T cell clone and irradiated PBMC and proliferative responses were assessed 48 h later. *B*, Recognition of *m*/*p*-BZ-(C)-C_5-OPP compounds is influenced by the spacing of the C_5-OPP from the BZ group. *m*-BZ-C-C_5-OPP ether (●) or its isomer *p*-BZ-C-C_5-OPP ether (○) and IPP (■), were added at serial half-log dilutions for stimulation of 12G12 with Va2 APC. *C*, Recognition of farnesyl and geranyl pyrophosphate BZ and DATFP photoaffinity compounds. Various FPP (◇, □, and △) and GPP (● and ○) compounds were tested for stimulation of 12G12 in the presence of Va2 APC. *D*, Recognition of *m*-BZ-C_5-OPP is dependent upon the presence of the pyrophosphate moiety. BZ (■), 4-maleimido-BZ (▲), or *m*-BZ-C_5-OPP (●) were added at half-log serial dilutions. An EBV transformed B cell line, DG.EBV, was used as the APC for the 12G12 T cell clone.

Vγ2Vδ2 T cells, *m*-BZ-C_5-OPP and *p*-BZ-C_5-OPP were tested for their ability to induce proliferation of Vγ2Vδ2 T cells. Similar to IPP and EPP, both compounds stimulated DG.SF68 Vγ2Vδ2 T cells in a dose-dependent manner (Fig. 2*A*). The concentrations that induced half-maximum proliferation were 0.4 μM, intermediate between IPP (1–3 μM) and HMBPP (0.0000316 μM). Thus, as predicted based on their structure, both *m*-BZ-C_5-OPP and *p*-BZ-C_5-OPP are recognized as Ags by Vγ2Vδ2 T cells.

To determine the specificity of recognition by Vγ2Vδ2 T cells, other photoaffinity analogues (both *m*- and *p*-substituted) with ether and ester linkages to the benzophenone group were tested (Fig. 2*B*, *C*, 3*C*, *D*). The linkage and spacing of the C_5-OPP group from the BZ group was extremely important in determining bioactivity. Compounds that had ester-linked C_5-OPP groups spaced one methylene group away from the BZ moiety (*m*/*p*-BZ-C-C_5-OPP esters) were extremely active, requiring only slightly higher concentrations for half-maximum stimulation compared with HMBPP (the most potent prenyl pyrophosphate described) with half-maximum stimulation at 50–60 pM vs 32 pM for HMBPP (see Figs. 3*C* and 4*C* and Ref. 3). Changing the ester linkage to an ether linkage (*m*/*p*-BZ-C-C_5-OPP ethers) reduced bioactivity by 38- to 72-fold (Fig. 3*C*). Removing the methylene spacer (to give *m*/*p*-BZ-C_5-OPP ethers) further reduced bioactivity by 140–316-fold (Fig. 2*A* vs 3*C*). Vγ2Vδ2 T cells showed equal or slight preferential recognition of *para*-BZ-(C)-C_5-OPP compounds compared with their *meta*- isomers (Figs. 2–4). This was most pronounced for *p*-BZ-C-GPP and *m*-BZ-C-GPP, compounds that differed by ~10-fold in activity (Fig. 2*C*). Compounds with DATFP photoaffinity groups attached to longer chain FPP and GPP moieties or with the BZ group linked to FPP via the pyrophosphate moiety had little or no activity (Fig. 2*C*). These results show that Vγ2Vδ2 T cells recognize isoprenoid photoaffinity compounds in a structure-specific manner and that the linkage to and spacing from the BZ moiety determine bioactivity levels.

Because recognition of prenyl pyrophosphate Ags by Vγ2Vδ2 T cells requires the pyrophosphate moiety, we sought to determine whether recognition of *m*-BZ-C_5-OPP shows a similar requirement. Neither the crosslinked BZ photophore without the pyrophosphate moiety nor a 4-maleimido-BZ derivative stimulated Vγ2Vδ2 T cells (Fig. 2*D*), suggesting that Vγ2Vδ2 T cell recognition of *m*-BZ-C_5-OPP is dependent on the presence of the pyrophosphate moiety and not the BZ group.

m-BZ-C_5-OPP can stably associate with APC after photocrosslinking and are presented directly like prenyl pyrophosphates

Previously, we and others have found that prenyl pyrophosphate Ags, including IPP and mono-ethyl phosphate, do not stably associate with APC (24, 26). To determine whether the FPP photoaffinity analog *m*-BZ-C_5-OPP can stably associate with APC after UV crosslinking, we incubated APC in medium only, with IPP, or with *m*-BZ-C_5-OPP for 90 min on ice. During this incubation, the cells were either exposed to UV light to induce crosslinking of *m*-BZ-C_5-OPP or left unexposed. After extensive washing, the cells were then used as APC to stimulate the 12G12 Vγ2Vδ2 T cell clone. Unlike APC pulsed with *m*-BZ-C_5-OPP (and not exposed to UV light), APC exposed to UV light during pulsing stimulated Vγ2Vδ2 T cells to proliferate even after extensive washing (Fig. 3*A*). UV treatment alone did not affect the APC, because APC exposed to UV light in either the absence or presence of IPP did not stimulate Vγ2Vδ2 T cells. The cells were competent for presentation because they were able to present *m*-BZ-C_5-OPP when the Ag was continuously present (Fig. 3*A*).

This recognition of *m*-BZ-C_5-OPP on DG.EBV B cells was dose and UV dependent. Even after extensive washing, the APC with UV-crosslinked *m*-BZ-C_5-OPP retained the ability to stimulate the CD4$^+$ Vγ2Vδ2 T cell clone HF.2 to proliferate in a dose-dependent fashion, whereas non-UV-crosslinked *m*-BZ-C_5-OPP did not (Fig. 3*B*). Similarly, UV crosslinking of the *m*/*p*-BZ-C-C_5-OPP ether- and ester-linked compounds also resulted in stable association with the APC that was resistant to washing (Fig. 3*C*). Crosslinked Ags required somewhat higher concentrations for half-maximal stimulation compared with Ag present continuously (~33-fold and ~13-fold higher for the ester and ether compounds, respectively) (Fig. 3*C*). Besides stimulating proliferative

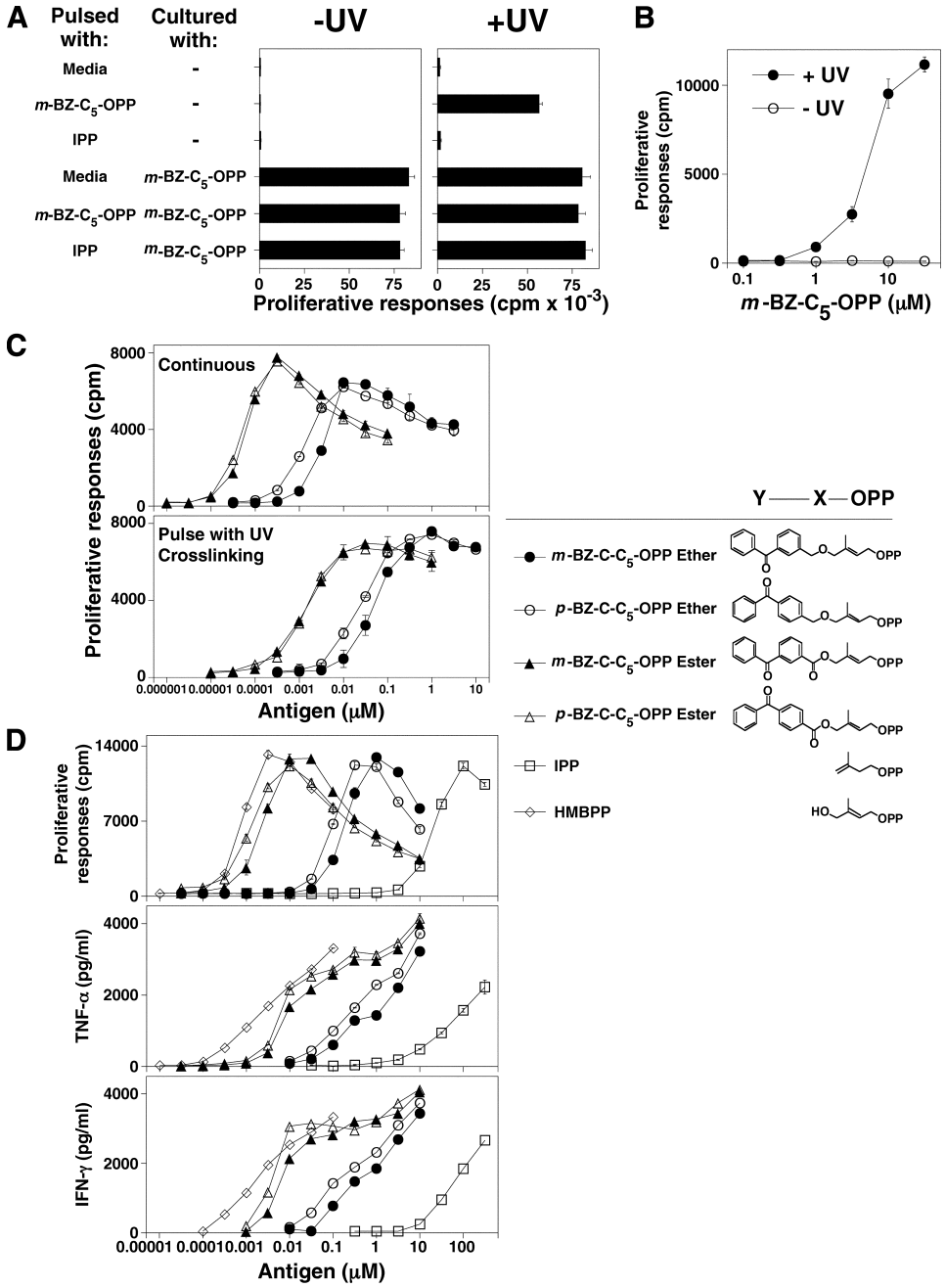

FIGURE 3. *m*-BZ-C_5-OPP can be crosslinked onto the surface of APC by UV light for stimulation of Vγ2Vδ2 T cells. *A*, *m*-BZ-C_5-OPP stably associates with the APC after UV crosslinking. Mitomycin C-treated DG.EBV B cells were incubated with medium, 5 μM *m*-BZ-C_5-OPP, or 250 μM IPP with or without UV crosslinking for 90 min on ice and washed three times. DG.EBV B cells (7.5×10^4) were cultured with 1×10^5 $CD4^+$ HF.2 Vγ2Vδ2 T cells in the continuous presence or absence of 10 μM *m*-BZ-C_5-OPP. After 24 h the cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 h later. *B*, Dose-dependent Vγ2Vδ2 T cell recognition of UV crosslinked *m*-BZ-C_5-OPP. Mitomycin C-treated DG.EBV B cells were incubated with varying concentrations of *m*-BZ-C_5-OPP with (●) or without (○) UV crosslinking for 90 min on ice followed by washing. HF.2 was then added to the washed APC and proliferation was determined by [^{3}H]thymidine incorporation. *C*, UV-crosslinked *m*/*p*-BZ-C-C_5-OPP ether and ester compounds stimulate Vγ2Vδ2 T cells. *m*/*p*-BZ-C-C_5-OPP compounds were tested with and without UV crosslinking for their ability to stimulate 12G12 when presented by the Va2 cell line. Pulsing was done in PBS without serum followed by washing four times in a 96-well plate. *D*, Photoaffinity compounds stimulate secretion of IFN-γ (*bottom panel*) and TNF-α (*middle panel*) and proliferation (*top panel*) by Vγ2Vδ2 T cells. *m*/*p*-BZ-C-C_5-OPP compounds, IPP, or HMBPP were incubated with the HF.2 T cell clone with DG.EBV APC. After 16 h, culture supernatants were harvested and tested for cytokines by ELISA. Proliferation was assessed by [^{3}H]thymidine incorporation.

responses, the photoaffinity Ags also stimulated the release of TNF-α and IFN-γ in a dose-dependent manner (Fig. 3*D*). These findings show that *m*/*p*-BZ-(C)-C_5-OPP compounds stimulate Vγ2Vδ2 T cell cytokine and proliferative responses and that these compounds retains their immunogenicity when crosslinked to the APC surface.

To determine the mechanism by which *m*/*p*-BZ-(C)-C_5-OPP compounds stimulate Vγ2Vδ2 T cells, we inhibited the response of Vγ2Vδ2 T cells with the statin, mevastatin. We found that compounds that act as direct Ags for Vγ2Vδ2 T cells (i.e., prenyl pyrophosphates) or as mitogens (e.g., PHA) are much less sensitive to statin inhibition than compounds (e.g., bisphosphonates and alkylamines) that act indirectly by inhibiting FPPS, causing IPP accumulation (H. Wang and C. T. Morita, manuscript in preparation). Therefore, we used mevastatin to inhibit Vγ2Vδ2 T cell responses induced by *p*-BZ-C_5-OPP in comparison with HMBPP and risedronate. Whereas mevastatin inhibited 50% of the response to the FPPS inhibitor risedronate at 0.07 μM (Fig. 4*A*), mevastatin concentrations of 23–42 μM (328- to 600-fold higher) were required to inhibit *p*-BZ-C_5-OPP responses (Fig. 4, *A* and *B*). These levels were similar to the 80 μM mevastatin concentrations that were required to inhibit 50% of the HMBPP responses by the CD4 HF.2 clone (HMBPP presented by CP.EBV) or the CD8αα 12G12 clone (HMBPP presented by Va2) (Fig. 4, *A* and *B*).

A second characteristic of prenyl pyrophosphate Ags is their recognition by Vγ2Vδ2 T cells in the absence of other cells due to presentation by daughter T cells (24). In contrast, bisphosphonates generally require the presence of APC to stimulate proliferation of Vγ2Vδ2 T cells (37) (although this may be due in part to toxicity associated with the inhibition of FPPS). To determine whether the photoaffinity Ags require APC for presentation, the 12G12 and HD.108 Vγ2Vδ2 T cell clones were incubated with the *m*/*p*-BZ-C-C_5-OPP compounds IPP and HMBPP in the presence or absence of Va2 cells as APC. Like IPP and HMBPP, the *m*/*p*-BZ-C-C_5-

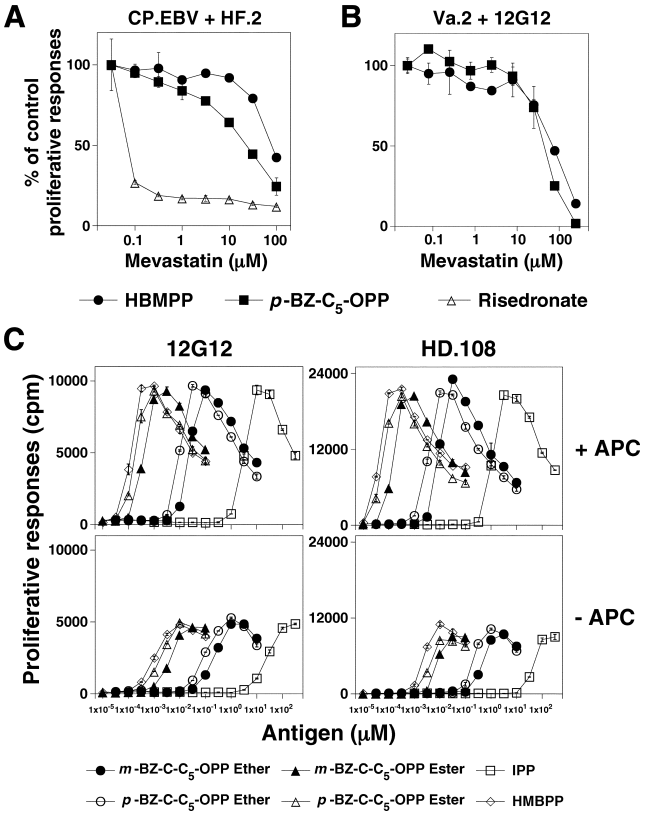

FIGURE 4. Photoaffinity Ags act as direct Ags for Vγ2Vδ2 T cells. *A*, Mevastatin inhibition of *p*-BZ-C_5-OPP is consistent with direct stimulation like a prenyl pyrophosphate Ag rather than indirect stimulation via FPPS inhibition. APC were incubated with varying concentrations of mevastatin for 30 min followed by the addition of *p*-BZ-C_5-OPP, the prenyl pyrophosphate, HMBPP, or the bisphosphonate, risedronate. For risedronate, the cells were washed after 1 h. $CD4^+$ HF.2 Vγ2Vδ2 T cells were then added to each culture and proliferative responses were determined as in Fig. 3*A*. Mean maximal proliferative responses for *p*-BZ-C_5-OPP, HMBPP, and risedronate were 14,545 ± 323, 2,738 ± 7, and 3,342 ± 846, respectively. *B*, Inhibition of *p*-BZ-C_5-OPP and HMBPP proliferative responses by mevastatin. The Va2 cell line was incubated with mevastatin for 30 min followed by the addition of *p*-BZ-C_5-OPP or HMBPP and the 12G12 cell clone. Proliferative responses were determined as in Fig. 3*A*. Mean maximal proliferative responses for *p*-BZ-C_5-OPP and HMBPP were 28,974 ± 495 and 15,863 ± 1,099, respectively. *C*, Vγ2Vδ2 T cells respond to *m*/*p*-BZ-(C)-C_5-OPP compounds in the absence of other accessory/presenter cells. *m*/*p*-BZ-(C)-C_5-OPP compounds, IPP, and HMBPP were used to stimulate proliferation of 12G12 and HD.108 Vγ2Vδ2 T cell clones in the presence (*top panels*) or absence (*bottom panels*) of Va2 presenter cells.

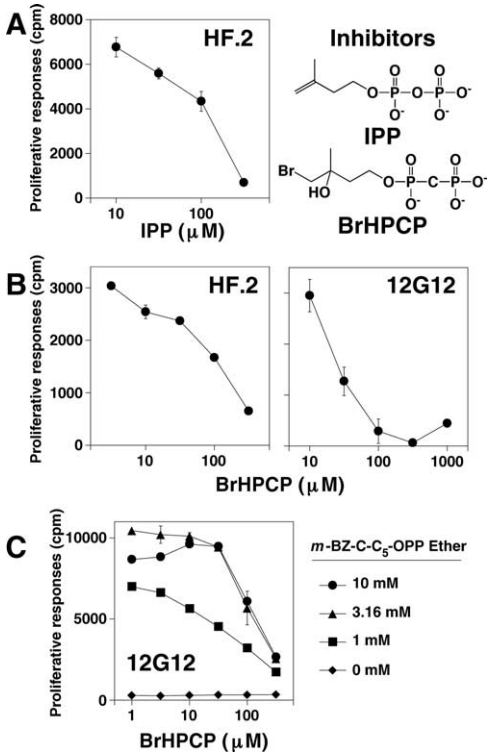

FIGURE 5. IPP and the inactive BrHPP analog BrHPCP inhibit pulsing and recognition of *m*-BZ-(C)-C_5-OPP by Vγ2Vδ2 T cells. *A*, IPP inhibits the crosslinking of *m*-BZ-C_5-OPP to the APC surface. Mitomycin C-treated, EBV-transformed B cells (DG.EBV) in P-medium with serum were incubated with varying concentrations of IPP for 30 min after which 0.316 μM *m*-BZ-C_5-OPP was added and the APC were further incubated for 90 min on ice with UV exposure. Following crosslinking, the APC were washed extensively and 1×10^5 APC were cultured with 1×10^5 HF.2 T cells. After 24 h the cultures were pulsed with 1 μCi [^{3}H]thymidine and harvested 18 h later. *B*, BrHPCP inhibits the crosslinking of *m*-BZ-C-C_5-OPP ether to APC. BrHPCP (structure detailed in *A*) was incubated with DG.EBV followed by the addition of 1 μM *m*-BZ-C-C_5-OPP ether and treatment with UV light for crosslinking. Unbound Ag was washed away and the APC were cultured with HF.2 (*left panel*) or 12G12 T cells (*right panel*) as detailed in *A*, except that background proliferation was subtracted for 12G12 with DG.EBV. *C*, BrHPCP inhibits Vγ2Vδ2 T cell responses to *m*-BZ-C-C_5-OPP ether. Va2 APC were incubated with different concentrations of BrHPCP followed by the addition of varying concentrations of *m*-BZ-C-C_5-OPP ether in continuous culture. 12G12 Vγ2Vδ2 T cells were then added and proliferation was determined as detailed in *A*. Note that BrHPCP alone (♦) did not induce 12G12 proliferation.

OPP photoaffinity compounds stimulated Vγ2Vδ2 T cell proliferation in the complete absence of APC with similar lower magnitude responses and shifted dose-response curves (Fig. 4*C*). Thus, photoaffinity compounds function as direct Ags for Vγ2Vδ2 T cells rather than as pharmacological inhibitors of FPPS.

IPP and the BrHPCP analog inhibit pulsing and recognition of m-BZ-(C)-C_5-OPP by Vγ2Vδ2 T cells

Because both IPP and *m*-BZ-C_5-OPP induce the proliferation of Vγ2Vδ2 T cells, we sought to determine whether they bind to the same sites on the APC surface. To test this, APC were preincubated with varying concentrations of IPP and then 0.316 μM *m*-BZ-C_5-OPP was added and the cells exposed to UV light. Following UV treatment, the APC were washed and tested for their ability to stimulate Vγ2Vδ2 T cells. When no competing Ag was present, *m*-BZ-C_5-OPP crosslinked efficiently to the surface of DG.EBV and induced proliferation of HF.2 Vγ2Vδ2 T cells (Fig. 5*A*). However, preincubation with IPP inhibited *m*-BZ-C_5-OPP crosslinking to the APC in a dose-dependent fashion, decreasing their ability to stimulate HF.2 T cells. Similarly, the inactive bromohydrin pyrophosphate analog, BrHPCP (structure shown in Fig. 5*A*) blocked crosslinking of the *m*-BZ-C-C_5-OPP ether Ag to the APC surface as evidenced by the inhibition of the proliferation of the HF.2 and 12G12 clones with increasing concentrations of BrHPCP (Fig. 5*B*). The continuous presence of BrHPCP also inhibited stimulation by the *m*-BZ-C-C_5-OPP ether Ag even when not crosslinked (Fig. 5*C*), confirming an earlier study (20). Note that BrHPCP did not stimulate Vγ2Vδ2 T cells to proliferate or secrete TNF-α nor was there any evidence for direct toxicity (Fig. 5*C* and data not shown). These findings demonstrate that IPP/BrHPCP compete with *m*-BZ-(C)-C_5-OPP Ags for binding sites on the APC cell surface, preventing stable crosslinking and presentation.

Table I. *Human but not murine tumor cells from a variety of cell lineages serve as antigen presenting cells for m-BZ-C_5-OPP/prenyl pyrophosphates*

Cell Line/Lineage	Designation	Tumor Type	*m*-BZ-C_5-OPP/Prenyl Pyrophosphate Presentation
Human Cell Lines[a]			
Hematopoietic			
B cell	DG.EBV	EBV transformed	++
B cell	721	EBV transformed	++
B cell	Raji	Burkitt's lymphoma	++
T cell	J.RT3-T3.5	Thymoma	++
Myeloid cell	K562	Erythroleukemia	++
Myeloid cell	U-937	Myelomonocytic Leukemia	++
Myeloid cell	THP-1	Monocytic Leukemia	+
Nonhematopoietic			
Epithelial cell	T84	Colon Adenocarcinoma	+/−
Epithelial cell	HT-29	Colon Adenocarcinoma	+
Epithelial cell	HuTu 80	Duodenal Adenocarcinoma	++
Epithelial cell	JAR	Choriocarcinoma	+
Epithelial cell	HeLa	Cervical carcinoma (Epitheloid)	++
Epithelial cell	A-431	Skin (Epidermoid)	+
Melanocyte	FO-1	Melanoma	++
Melanocyte	SK-MEL-28	Melanoma	++
Neural crest	SH-5YSY	Neuroblastoma	++
Fibroblast	HT-1080	Fibrosarcoma	+++
Fibroblast	Va2	Transformed fibroblast	+++
Fibroblast	IMR-90	Fibroblast	+
Squamous epithelium	FADU	Squamous cell carcinoma	++
Squamous epithelium	SCC24a	Squamous cell carcinoma	++
Squamous epithelium	CAL 27	Squamous cell carcinoma	++
Squamous epithelium	DV	Squamous cell carcinoma	++
Murine cell lines[b]			
Hematopoietic			
B cell	A20	B cell Lymphoma	−
T cell	EL4	T cell lymphoma	−
Dendritic cell	DC2.4	Immortalized dendritic cell	−
Monocyte/macrophage	J774	Sarcoma	−
Macrophage	RAW 264.7	A-MuLV transformed	−
Mast cell	P815	Mastocytoma	−

[a] Assessed by stimulation of proliferation of Vγ2Vδ2 T cell clones after UV crosslinking.
[b] Assessed by pulsing or culturing murine cell lines with *m*-BZ-C_5-OPP, IPP, HMBPP, and/or risedronate and using as APCs to stimulate the proliferation of Vγ2Vδ2T cell clones.

Photoaffinity Ags can be presented by a broad array of hematopoietic and nonhematopoietic tumor cell lines

The ability to covalently attach the prenyl pyrophosphate analog to a molecule on the APC surface allowed us to test tumor cells from a variety of different lineages for expression of the presenting molecule without the complicating possibility of self-presentation by Vγ2Vδ2 T cells to each other. We found that virtually any human tumor cell line, irrespective of tissue origin or developmental stage, was able to present the *m*-BZ-C_5-OPP Ag to Vγ2Vδ2 T cells (Table I). In contrast, none of the six murine hematopoietic cell lines tested were able to present HMBPP or the bisphosphonate risedronate to Vγ2Vδ2 T cells (Table I). This suggests that the putative Ag-presenting molecule for prenyl pyrophosphate Ags is broadly distributed like classical MHC class I molecules but is functionally nonpolymorphic.

Prenyl pyrophosphate Ags can be pulsed onto APC without crosslinking

During our survey of different tumor cell lines, we noted some, such as Va2, SH-5YSY, and HT-1080, that presented pulsed *m*-BZ-C_5-OPP without UV crosslinking (Fig. 6*A*). When pulsed in RPMI 1640 with FCS, the Va2 cell line was able to present *m*-BZ-C_5-OPP without UV crosslinking but required a 200-fold higher concentration during pulsing to elicit a similar half-maximal response to that observed in the continuous presence of the Ag (0.21 vs 42 μM; Fig. 6*B*). IPP could also be pulsed onto Va2 but required a 100-fold higher concentration to elicit a similar level of response to the continuous presence of the Ag (Fig. 6*B*). Thus, prenyl pyrophosphate Ags can be pulsed onto APC without covalent linkage, albeit inefficiently under standard conditions.

In earlier attempts at pulsing prenyl pyrophosphates, we used EBV-transformed B cells or PBMC as APC with moderate potency Ags such as IPP, mono-methyl-phosphate, EPP, or low concentrations of HMBPP in bacterial lysates (24). Because APC may differ in their ability to present prenyl pyrophosphates, we compared the ability of three tumor cell lines (the EBV-transformed B cell line, DG.EBV, Va2, and SH-5YSY) to present *m*-BZ-C_5-OPP (without UV crosslinking) to the NKG2D$^+$CD8αα$^+$ Vγ2Vδ2 T cell clone DG.SF68 (Fig. 6*C*).

DG.EBV B cells stimulated Vγ2Vδ2 T cell proliferative responses similar to or higher than those of the other presenter cell lines. However, compared with Va2, DG.EBV cells required Ag concentrations for half-maximal responses (at the optimal APC number) that were 17-fold higher for mono-ethyl phosphate (Expt. 1) and 12-fold higher for IPP (Expt. 2) (Fig. 6*C*). The concentrations required by SH-5YSY were intermediate between the two cell lines. Thus, the Va2 and SH-5YSY cell lines were more effective presenter cells than was DG.EBV.

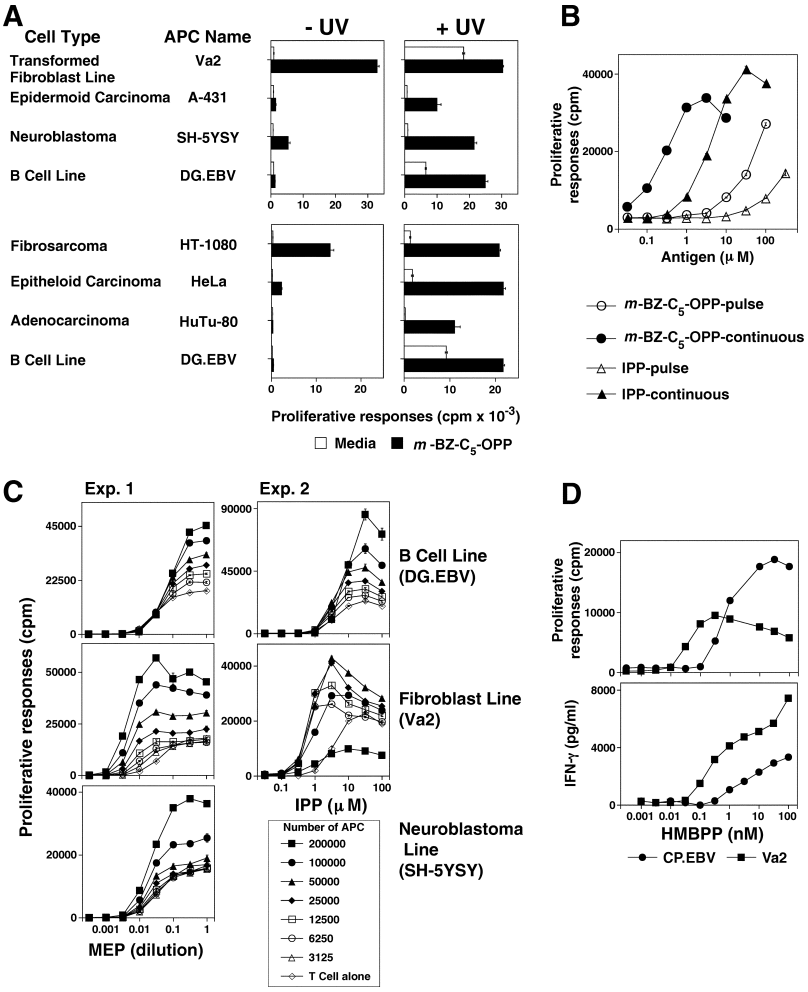

FIGURE 6. Prenyl pyrophosphate Ags can be pulsed onto APC without UV crosslinking. *A*, Some cell lines stably associate with *m*-BZ-C_5-OPP without UV treatment. Various mitomycin C-treated tumor cell lines were incubated for 90 min with medium alone or medium with 40 μM *m*-BZ-C_5-OPP in the presence or absence of 350 nm UV light on ice. The APC were then washed three times with PBS at 4°C and 1×10^5 APC were cultured with 1×10^5 12G12 T cells in medium. After 24 h the cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 h later. Note that the Va2, SH-5YSY, HT-1080, and HeLa cell lines pulsed with *m*-BZ-C_5-OPP stimulate the 12G12 T cells even without UV crosslinking with varying efficiency. *B*, Efficient presentation by the Va2 cell line reveals pulsing of prenyl pyrophosphates. For continuous exposure, *m*-BZ-C_5-OPP (●) or IPP (▲) were added directly to culture. For pulsing, the transformed fibroblast line Va2 was treated with mitomycin C and incubated with *m*-BZ-C_5-OPP (○) or IPP (△) for 1 h in P-medium with FCS in the absence of UV treatment on ice. The APC were washed seven times in PBS at 4°C and then incubated with the CD8$\alpha\alpha^+$ NKG2D$^+$ 12G12 T cell clone. *C*, Efficient presentation of prenyl pyrophosphate Ags by some tumor cell lines to the CD8$\alpha\alpha^+$ NKG2D$^+$ DG.SF68 T cell clone. Varying concentrations of the Va2, SH-5YSY, and DG.EBV cells were continuously cultured with DG.SF68 T cells with varying dilutions of mono-ethyl-phosphate (MEP) or IPP. *D*, Efficient presentation of prenyl pyrophosphate Ags by the Va2 cell line to the CD4$^+$ NKG2D HF.2 T cell clone. Mitomycin C-treated Va2 or CP.EBV cells were cultured with the CD4$^+$ HF.2 T cell clone in the presence of HMBPP. To assess IFN-γ levels, supernatants were harvested at 24 h and IFN-γ levels were determined by ELISA. Proliferation was determined as in *A*.

Va2 are more effective presenter cells, in part because they express the MICA, ULBP2, and ULBP3 ligands that bind to the NKG2D receptors expressed on the surface of the CD8 $\alpha\alpha^+$ Vγ2Vδ2 DG.SF68 T cell clone. We have previously shown that NKG2D binding to its ligands enhances Vγ2Vδ2 T cell responses to prenyl pyrophosphates (12) and that DG.EBV lacks such NKG2D ligands (data not shown). However, there are likely additional accessory molecule interactions or other factors that enhance recognition, because the CD4$^+$ Vγ2Vδ2 T cell clone HF.2 (which lacks NKG2D) also requires 16-fold higher HMBPP concentrations with DG.EBV as compared with Va2 (half-maximal proliferation at 0.53 vs 0.033 nM for DG.EBV vs Va2; Fig. 6*D*). Thus, prenyl pyrophosphate Ags can be pulsed onto APC, although this is difficult to demonstrate using IPP and B cells.

m-BZ-C_5-OPP pulsing is inhibited by serum and medium components

Because prenyl pyrophosphates can pulse onto APC, albeit inefficiently, even without UV crosslinking (Fig. 6), we sought to optimize conditions for the pulsing of *m*-BZ-C_5-OPP onto APC. Unlike earlier experiments where medium with serum was used (Fig. 6*B*), we pulsed *m*-BZ-C_5-OPP onto APC in PBS either with or without serum in the presence or absence of UV light to crosslink the Ag (Fig. 7). Following pulsing, the APC were washed and resuspended in medium with serum and cultured with the 12G12 clone. To rule out changes in the APC due to UV exposure or the lack of serum, control APC were treated as above in the absence of *m*-BZ-C_5-OPP and then assessed for their ability to present *m*-BZ-C_5-OPP added with the T cells (Fig. 7*A*, *bottom panels*). In the absence of serum, pulsing of *m*-BZ-C_5-OPP was very efficient following crosslinking to the APC (Fig. 7*A*, *top right panel*). Importantly, even without UV crosslinking, *m*-BZ-C_5-OPP could be pulsed onto the APC with similar efficiency (0.33 vs 0.20 μM respectively; Fig. 7*A*, *top left panel* vs *top right panel*). Thus, pulsing was more efficient in the absence of serum, and this effect was more pronounced for *m*-BZ-C_5-OPP that had not been crosslinked (21-fold for uncrosslinked vs 5-fold for UV-crosslinked). These results suggest that an unknown serum component inhibits the efficient pulsing of *m*-BZ-C_5-OPP onto APC.

Because pulsing of *m*-BZ-C_5-OPP onto APC was most efficient when the pulsing reaction was conducted without serum, we extended these observations to natural prenyl pyrophosphate Ags. We incubated HMBPP in either medium with or without serum or in PBS without serum (Fig. 7*B*). When HMBPP was continuously present, serum had little effect on Vγ2Vδ2 T cell proliferation (Fig. 7*B*, *left bottom panel*). However, pulsing of HMBPP onto APC was better in medium without serum and better still in PBS without serum (Fig. 7*B*, *top panels*). Thus, pulsing of both natural and synthetic prenyl pyrophosphates onto APC is inhibited by serum and by other medium components.

Prenyl pyrophosphate Ags pulse rapidly onto APC

Because we found that prenyl pyrophosphate Ags could be pulsed efficiently onto APC, we sought to determine the kinetics of nonpeptide Ag pulsing. Mitomycin C-treated or glutaraldehyde-fixed

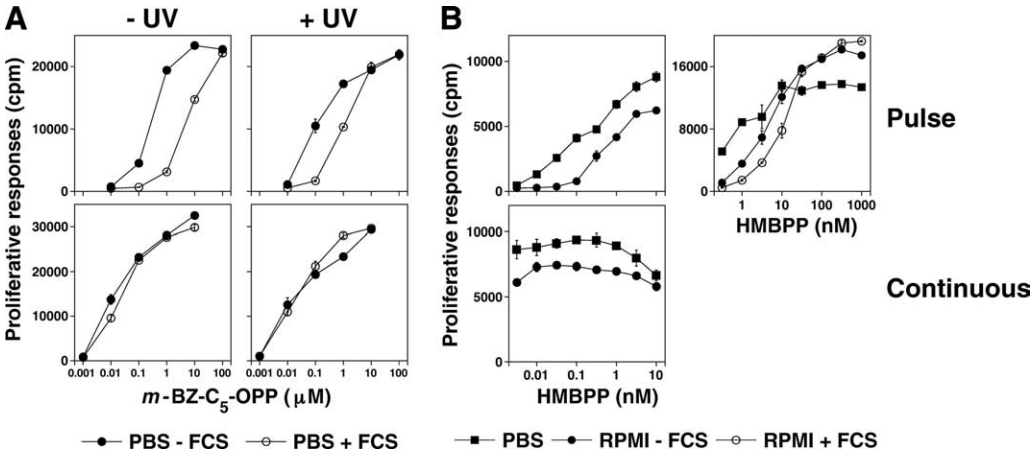

FIGURE 7. Stable association of *m*-BZ-C_5-OPP and other prenyl pyrophosphates with APC is impaired by serum. *A*, *m*-BZ-C_5-OPP pulsing is impaired by a serum component. Mitomycin C-treated Va2 cells were resuspended in PBS with (○) or without FCS (●) and incubated with or without *m*-BZ-C_5-OPP in the presence (+) or absence (−) of UV light for 90 min on ice. The APC were then washed three times with PBS at 4°C. Va2 cells (4×10^4) that had been pulsed with Ag (*top panels*) or not pulsed (*bottom panels*) were then cultured with 1×10^5 DG.SF68 Vγ2Vδ2 T cells. For the nonpulsed APC, *m*-BZ-C_5-OPP was continuously present. To assess proliferation, the cultures were pulsed with 1 μCi of [^{3}H]thymidine at 24 h and harvested 18 h later. *B*, HMBPP pulsing is impaired by compounds in serum and RPMI 1640 medium. Va2 was treated with mitomycin C, resuspended in PBS (■) or RPMI 1640 medium without (●) or with (○) serum and incubated with varying concentrations of HMBPP for 1 h at 37°C. The APC were washed three times in PBS at room temperature, resuspended in medium with serum, and incubated with the 12G12 CD8αα$^+$ Vγ2Vδ2 T cell clone. As a control, HMBPP was added back to the APC for continuous culture with γδ T cells.

Va2 cells were incubated with HMBPP for varying lengths of time (5–120 min) in PBS without serum after which the APC were washed extensively. The pulsed APC were then incubated with the CD4$^+$ HF.2 clone and γδ T cell proliferation (Fig. 8) and release of TNF-α (data not shown) were measured. In agreement with our previous observations (24), fixing APC with glutaraldehyde had no affect on pulsing of HMBPP. In fact, glutaraldehyde-fixed APC were better than mitomycin C-treated APC at presenting nonpeptide Ags to Vγ2Vδ2 T cells (Fig. 8). Within 5 min of incubation with the prenyl pyrophosphate Ag HMBPP, ~75% of the antigenic activity of HMBPP was already associated with the APC. Pulsing of HMBPP onto glutaraldehyde-fixed APC peaked at 45 min of incubation, whereas mitomycin C-treated APC required 60–90 min of incubation. These results demonstrate that the putative Ag-presenting molecule on the APC associates with prenyl pyrophosphates very rapidly (<5 min). These data are consistent with the rapid activation of γδ T cells that we and others have observed (24–26) and with our IPP binding results (data not shown).

Recognition of prenyl pyrophosphates does not require APC expression of classical MHC class I, MHC class II, β_2M dependent, or CD1 molecules

We earlier demonstrated that Vγ2Vδ2 T cells do not require prenyl pyrophosphates to be internalized or processed for presentation and do not require professional APC (24). To determine whether a known Ag-presenting molecule was required for presentation of prenyl pyrophosphates, we tested mutant APC (24) that lacked these molecules and found their absence on the APC had no effect on prenyl pyrophosphate recognition and that mAbs to these molecules did not inhibit prenyl pyrophosphate recognition (55). However, because Vγ2Vδ2 T cells are as efficient as dendritic cells at presenting peptide Ags to αβ T cells (56) and can present prenyl pyrophosphates to each other (24), we could not exclude that a known Ag-presenting molecule on the Vγ2Vδ2 T cells themselves was presenting to daughter T cells in these experiments. To exclude presentation by Vγ2Vδ2 T cells to each other, we directly crosslinked *m*-BZ-C_5-OPP to different APC cell lines that lack expression of known Ag-presenting molecules and used the crosslinked APC to stimulate Vγ2Vδ2 T cells. In this way, we could avoid any possible presentation by Vγ2Vδ2 T cells, because the Ags are covalently linked to the APC. We found that MHC class II-negative APC (24), including the transcription factor mutant RAJI Burkitt's lymphoma cell line, RJ-2.2.5, the J.RT3-T3.5

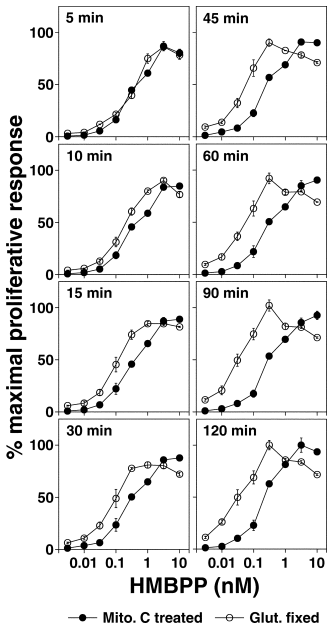

FIGURE 8. Prenyl pyrophosphate Ags pulse rapidly onto the APC cell surface. The Va2 cell line was treated with mitomycin C (Mito. C) (●) or fixed with glutaraldehyde (Glut.) (○) and incubated at 37°C with HMBPP for the indicated time period. The cells were then washed three times in PBS at room temperature, resuspended in medium with serum, and incubated with the CD8αα$^+$ Vγ2Vδ2 T cell clone 12G12 for 48 h. The highest proliferative response at 120 min was normalized to 100% maximal proliferative activity. Note that HMBPP pulses onto APC very rapidly in PBS, with ~75% of maximal antigenic activity associating with the APC at the earliest time point (5 min).

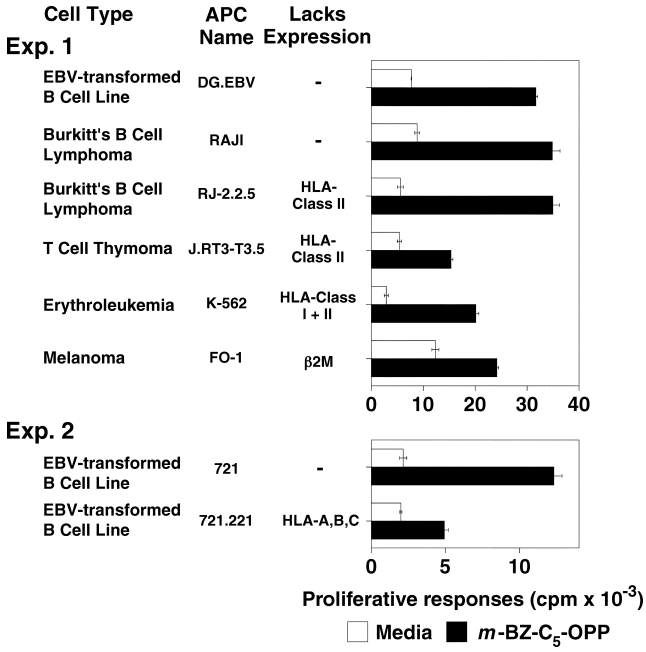

FIGURE 9. Expression of classical MHC class I molecules, β_2M-dependent molecules, MHC class II molecules, and CD1 molecules is not required for the presentation of prenyl pyrophosphate Ags to Vγ2Vδ2 T cells. The HF.2 Vγ2Vδ2 T cell clone was cultured with (Expt. 1) the EBV-transformed B cell line DG.EBV, the Burkitt's lymphoma cell line Raji and its mutant derivative line RJ-2.2.5 (lacking MHC class II), the erythroleukemia cell line K562 (lacking MHC class I and class II), the TCRβ^- thymoma cell line J.RT3-T3.5 (lacking MHC class II), and the melanoma cell line FO-1, (lacking β_2M) or with (Expt. 2) the parent EBV-transformed B cell line 721 and its mutant 721.221 (lacking classical HLA-A, -B, and -C MHC class I) in the absence (open bars) or presence (filled bars) of UV-crosslinked *m*-BZ-C_5-OPP Ag.

thymoma, and the erythroleukemia K-562, were able to present crosslinked *m*-BZ-C_5-OPP to Vγ2Vδ2 T cells (Fig. 9). Similarly, 721.221, which lacks HLA-A, -B, and -C expression, the erythroleukemia cell line K-562, which lacks MHC class I, as well as the melanoma cell line FO-1, which lacks β_2M expression, all presented *m*-BZ-C_5-OPP to Vγ2Vδ2 T cells (Fig. 9). Also, expression of CD1a, CD1b, CD1c, and CD1d molecules was not required because the EBV-transformed B cell line DG.EBV and the Burkitt's cell line RAJI, both lack these molecules (57) yet still present *m*-BZ-C_5-OPP. These data clearly demonstrate, therefore, that recognition of prenyl pyrophosphate Ags by Vγ2Vδ2 T cells does not require classical MHC class I, MHC class II, β_2M, or CD1 expression by the APC.

Discussion

The molecular basis for prenyl pyrophosphate recognition by human Vγ2Vδ2 T cells is poorly understood due to the inability to identify an Ag-presenting molecule or to measure binding of the Vγ2Vδ2 TCR to these compounds. We sought photoaffinity prenyl pyrophosphate Ags that would stably crosslink to the APC surface to aid in these studies. To achieve this goal, we used *m*/*p*-BZ-(C)-C_5-OPP ester- and ether-linked photoaffinity analogues of FPP and HMBPP (40). We had previously found that *m*-BZ-C_5-OPP and *p*-BZ-C_5-OPP were substrates for three bacterial prenyl transferases and underwent efficient chain elongation to polyprenyl diphosphates (40). We report here that these compounds also stimulate Vγ2Vδ2 T cells at lower concentrations than IPP. Recognition of the *m*/*p*-BZ-(C)-C_5-OPP ester- and ether-linked photoaffinity compounds was greatly affected by the type of linkage and the spacing from the BZ moiety and required the presence of the pyrophosphate moiety. Based on statin sensitivity and APC independence, recognition of *m*/*p*-BZ-(C)-C_5-OPP was clearly due to their direct antigenic activity rather than any ability to inhibit FPPS. Importantly, *m*/*p*-BZ-(C)-C_5-OPP Ags retained immunogenicity even after UV crosslinking to the APC surface. IPP and a nonstimulatory pyrophosphonate analog of BrHPP, BrHPCP, blocked the covalent crosslinking of *m*-BZ-(C)-C_5-OPP to the APC cell surface, suggesting that they bind to the same sites on the APC as do the *m*-BZ-(C)-C_5-OPP Ags. *m*-BZ-C_5-OPP was able to stably associate with the cell surface of human hematopoietic and nonhematopoietic cell lines, including ones lacking known Ag-presenting molecules, for stimulation of Vγ2Vδ2 T cells. Thus, the molecule(s) that the photoaffinity Ags bind to are broadly distributed, functionally nonpolymorphic, and not a known Ag-presenting molecule.

Photoaffinity derivatives of Ags, GTP, and other ligands have been used to dissect various aspects of cellular functions and to define the binding of antigenic peptides to MHC class I. For instance, photoreactive derivatives of cyclosporins have been used to demonstrate the binding of cyclosporins to cyclophilins and the subsequent complex formation with calcineurin (58). Photoaffinity derivatives of antigenic peptides have been used to demonstrate that cell surface MHC class I glycoproteins do bind peptide Ags and that this interaction takes place even in the absence of the $\alpha\beta$ TCR (59). Further, a photoreactive derivative of the *Plasmodium berghei* antigenic peptide, *P.b.* CS 249–260, bound to cell-associated MHC class I molecules (60) and was used to determine the peptide binding motif for the H-2K^d molecule (61). The same photoreactive peptide has also been used to demonstrate that the avidity of TCR-ligand interactions is strengthened by CD8 on T cells (62) and that CD8β (but not CD8α) was involved in p56 binding in lipid rafts. Recently, it was used to demonstrate that the α-chain of the $\alpha\beta$ TCR is involved not just in binding to the ligand but is also involved in enhancing the CD8-TCR interaction (63). Other photoreactive probes have been used to identify the nucleotide binding sites in human IL-2 (64), GTP binding proteins that are biologically active in the T lymphocyte and thymocyte plasma membranes (65), and the active sites of enzymes. These studies demonstrate the usefulness of photoaffinity ligands/Ags to identify and isolate interacting or binding proteins.

For our study, we used BZ compounds that were originally developed as analogues of FPP (40). These compounds are photoactivatable substrates for isoprenoid pathway enzymes such as FPPS, farnesyl transferase, GPP synthase, and undecaprenyl pyrophosphate synthase and can label these enzymes. Because we had shown that Vγ2Vδ2 T cells recognize FPP (5, 24), we reasoned that these analogues might also be recognized. Indeed, *m*-BZ-C_5-OPP stimulated Vγ2Vδ2 T cells to proliferate like other prenyl pyrophosphates, even after photocrosslinking to the cell surface. This stimulation by *m*-BZ-C_5-OPP (which has a large aromatic BZ moiety at the end of the five-carbon alkenyl chain (Fig. 1)) is consistent with our finding that the carbon chain closest to the pyrophosphate moiety plays the critical role in determining Vγ2Vδ2 T cell stimulation (21). The type of linkage and spacing from the BZ group was very important in determining bioactivity, with the highest activity noted with ester linkage of the alkenyl pyrophosphate spaced one carbon from the BZ group. In many cases, Vγ2Vδ2 T cells could also distinguish between the *m* and *p* isomers of BZ-(C)-C_5-OPP compounds, similar to their ability to distinguish between the (*R*)- and (*S*)-stereoisomers of the chiral

phosphoantigens, BrHPP, and 3,4-epoxy-3-methyl-1-butyl pyrophosphate (66), and the (*E*)- and (*Z*)- forms of HMBPP (67). Recognition of *m*-BZ-C_5-OPP also requires the pyrophosphate moiety, because the BZ photophore and the 4-maleimide derivative of BZ (both lacking the pyrophosphate moiety) failed to stimulate Vγ2Vδ2 T cell proliferation. Thus, like synthetic and natural phosphoantigens (21), recognition of *m*/*p*-BZ-(C)-C_5-OPP Ags is critically dependent on the phosphate moiety and the adjacent alkenyl chain. Large moieties such as a BZ attached to the alkenyl chain or a ribonucleotide phosphate attached to the pyrophosphate group do not interfere with recognition if spaced sufficiently far away from the C_5-OPP structure.

Although, like bisphosphonates, *m*-BZ-C_5-OPP binds to FPPS, it has only very low activity as an inhibitor, requiring 250 μM for 20% inhibition of FPPS activity as compared with 20% stimulation of Vγ2Vδ2 T cells at a 2,500-fold lower concentration of 0.1 μM. Thus, it is likely to function as a direct Ag. Supporting this mechanism of stimulation of Vγ2Vδ2 T cells, the response of Vγ2Vδ2 T cells to *m*-BZ-C_5-OPP is highly resistant to mevastatin inhibition. This is identical to prenyl pyrophosphate responses (Fig. 4, *A* and *B*) and unlike bisphosphonate and alkylamine responses, which are very sensitive to statin inhibition (Fig. 4*A* and H. Wang and C. T. Morita, manuscript in preparation). The photoaffinity Ags can also stimulate Vγ2Vδ2 T cells in the absence of additional APC like prenyl pyrophosphates (Fig. 4*C* and Ref. 24). Thus, *m*/*p*-BZ-(C)-C_5-OPP Ags function as direct Ags for Vγ2Vδ2 T cells rather than as indirect stimulators through pharmacological inhibition of FPPS.

Although recognition of *m*-BZ-C_5-OPP by Vγ2Vδ2 T cells was specific and direct, it was not clear whether the BZ photophore was crosslinking specifically or nonspecifically to the cell surface. To address this question, we used IPP and the biologically inactive pyrophosphonate analog of BrHPP, BrHPCP, to compete with *m*-BZ-(C)-C_5-OPP Ags for binding to the APC surface. As expected if *m*-BZ-(C)-C_5-OPP and IPP/BrHPCP were competing for the same specific binding sites, the stimulatory activity of photocrosslinked *m*-BZ-(C)-C_5-OPP for γδ T cells was diminished in a dose-dependent manner by the presence of IPP or BrHPCP during UV crosslinking. This result strongly suggests that IPP and *m*-BZ-C_5-OPP compete for the same binding sites on the APC surface. Our results also would suggest an alternative explanation for the specific inhibitory activity of pyrophosphonate (methylene diphosphonate) and difluorodiphosphonate analogues of bromohydrin and iodohydrin pyrophosphate (20). Because BrHPCP prevents the crosslinking of *m*/*p*-BZ-(C)-C_5-OPP compounds (Fig. 5), it likely competes for the same binding sites on the cell surface as IPP. We speculate that rather than blocking dephosphorylation of phosphoantigens due to their nonhydrolyzable phosphonate bonds, these phosphonate compounds compete for binding with prenyl pyrophosphate Ags to the proposed presenting molecule. Unlike phosphoantigens, bound pyrophosphonate compounds are not recognized by the Vγ2Vδ2 TCR because of their structural differences from pyrophosphate compounds. Such inhibition of binding would be predicted to result in Ag-specific antagonism but to not affect Vγ2Vδ2 T cell mitogen responsiveness, identical to what was observed (20).

Prenyl pyrophosphates may bind to a plasma protein before their presentation at the APC cell surface. A soluble protein could bind IPP or HMBPP and inhibit presentation to limit Vγ2Vδ2 T cell responses. Alternatively, a soluble protein could enhance presentation by binding IPP or HMBPP and then transferring them to cell surface molecules for presentation. For example, apolipoprotein E binds the exogenous α-galactosyl ceramide lipid Ag for uptake and presentation by CD1d to αβ NKT cells (68). In this study, we found that binding of *m*-BZ-C_5-OPP to the APC, as measured by stimulation of γδ T cell proliferation, was inhibited by serum and by nonprotein components of RPMI 1640 medium. In the absence of serum and medium components, natural prenyl pyrophosphate Ags, which were earlier reported not to associate with the APC cell surface (24), could be shown to stably associate with APC. However, this association is not very efficient, because it required 100- to 1,000-fold more Ag during pulsing to achieve the same stimulation as that observed when the Ag was continuously present. These results suggest that unknown components of serum and medium can diminish the binding of the negatively charged prenyl pyrophosphate Ags to the APC surface. Apolipoprotein A1 has been proposed to bind to the Vγ2Vδ2 TCR to enhance recognition of the F1 ATPase β subunit (69). It is possible that this lipoprotein interferes with prenyl pyrophosphate Ag binding to the putative presenting molecule. Serum albumin binding of the hydrophobic alkenyl chain of prenyl pyrophosphate could also compete for binding. Alternatively, this inhibition could be due to dephosphorylation of the Ags by the alkaline phosphatase that is present in the serum, because incubation of BrHPP with cells resulted in hydrolysis of the pyrophosphate moiety presumably through the action of cell surface alkaline phosphatase (20). RPMI 1640 medium contains divalent cations, amino acids, and other compounds that are absent in PBS and that might interfere with the binding of pyrophosphate Ags to the APC surface.

In the absence of serum and medium components, we found that the binding of prenyl pyrophosphate Ags with APC was rapid, being detectable within 5 min (the least amount of time required for experimental manipulation) (Fig. 8). This binding of pyrophosphate Ags with the APC likely takes seconds because we found that [^{14}C]IPP binding with APC was extremely rapid, taking only 30 s (minimum time required for experimental manipulation) to achieve near-maximal binding. Although rapid, IPP binding showed very low affinity and was difficult to accurately measure (data not shown). It is unlikely that the prenyl pyrophosphate Ags require internalization for presentation, because they can be pulsed onto APC that have been fixed with glutaraldehyde, supporting our previous observations (24).

Most tumor cells of human origin can present prenyl pyrophosphate Ags to Vγ2Vδ2 T cells (Table I). These results, taken together with previous studies, might suggest that prenyl pyrophosphates associate nonspecifically with the APC surface for recognition. However, we and others have found that only APC of human origin can present nonpeptide prenyl pyrophosphate Ags to γδ T cells, because APC from mice and other species fail to stimulate Vγ2Vδ2 T cells (Table I and Refs. 28 and 30). Moreover, we now demonstrate that IPP and HMBPP can be pulsed onto the APC cell surface. Although the lack of presentation by xenogeneic cells could reflect species differences in accessory and/or costimulatory molecules (28), these results rule out the simple model where prenyl pyrophosphate Ags associate with the APC cell surface nonspecifically to stimulate Vγ2Vδ2 T cells.

Earlier studies could not rule out that Vγ2Vδ2 T cells were presenting nonpeptide Ags to daughter Vγ2Vδ2 T cells, because recognition required the continuous presence of Ag. Because we could covalently link *m*-BZ-C_5-OPP to the APC surface, human cell lines lacking known Ag-presenting molecules could be tested for presentation of *m*-BZ-C_5-OPP to Vγ2Vδ2 T cells in the absence of soluble Ag, thus ruling out Ag presentation by the Vγ2Vδ2 T cells. Using the *m*-BZ-C_5-OPP photoaffinity Ag, we find that Vγ2Vδ2 T cells do not require classical MHC class I (HLA-A, HLA-B, and HLA-C), MHC class II, or CD1a, CD1b, CD1c, or CD1d molecules on APC for prenyl pyrophosphate recognition. These findings suggest that a novel cell surface molecule

is functioning to present these Ags. However, this putative presenting molecule would be predicted to be widely distributed and nonpolymorphic, given that most tumor cells (except for those lacking accessory molecules) can present Ag to Vγ2Vδ2 T cells despite coming from different tissues and different individuals.

Further supporting the existence of a presenting molecule is the restriction of recognition of prenyl pyrophosphate Ags to Vγ2Vδ2 T cells. We have shown that recognition is TCR mediated because transfection of Vγ2Vδ2 TCR cDNAs into the TCR^- mutant of the αβ T cell tumor, Jurkat, confers responsiveness to prenyl pyrophosphate Ags (11) and because recognition is blocked by mAbs to the γδ TCR (39, 70). Moreover, only Vγ2Vδ2 γδ T cell clones respond to the prenyl pyrophosphate Ags (39, 71, 72). Mutation of the Vγ2Vδ2 TCR in the Vγ2 and Vδ2 CDR3 regions and other CDR can abolish prenyl pyrophosphate recognition while preserving anti-TCR mAb responses (H. Wang and C. T. Morita, manuscript in preparation and Refs. 73–75). However, there is no evidence for direct binding to prenyl pyrophosphates to soluble Vγ2Vδ2 TCR (data not shown and 19). Also, unlike murine γδ TCR recognition of T22 MHC class Ib molecules (76), there is no conserved amino acid motif in the Vδ2 CDR3 region that could mediate Ag binding. These results, coupled with the small size of phosphoantigens (minimum recognition unit is methyl phosphate (21)), support the existence of an Ag-presenting molecule.

Among the various stimulating compounds for γδ T cells, we hypothesize that only prenyl pyrophosphates are directly presented on the APC cell surface to the Vγ2Vδ2 TCR. Supporting this assertion, prenyl pyrophosphate recognition can be extremely rapid (10 s) (24, 25) and is not abolished by glutaraldehyde fixation of the APC (24). In contrast, we and others have found that stimulation of human Vγ2Vδ2 T cells by bisphosphonates (14–16), alkylamines (77, 78), and certain tumor cells (16) is indirect and mediated by the intracellular accumulation of IPP. However, it is unclear how this intracellular IPP is detected at the cell surface. We speculate that there exists an intracellular pathway where the putative Ag-presenting molecule encounters IPP (and perhaps HMBPP from intracellular pathogens) in the cell, leading to their transport to the cell surface. Evidence that this pathway uses transport by multidrug-related protein 5 transport has recently been reported (79). The ability to covalently attach a prenyl pyrophosphate analog to a molecule on the APC surface using photocrosslinking is, therefore, a significant advance and should assist in identifying this putative Ag-presenting molecule for Vγ2Vδ2 T cells.

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Disclosures

The authors have no financial conflict of interest.

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