Preferential recognition of a microbial metabolite by human Vγ2Vδ2 T cells

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Abstract

Human Vγ2Vδ2 T cells are stimulated by prenyl pyrophosphates, such as isopentenyl pyrophosphate (IPP), and play important roles in mediating immunity against microbial pathogens and have potent anti-tumor activity. (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) has been identified as a metabolite in the 2-C-methyl-D-erythritol-4 phosphate (MEP) pathway for isoprenoid biosynthesis that is used by many bacteria and protozoan parasites. We find that HMBPP is the major Vγ2Vδ2 T-cell antigen for many bacteria, including Mycobacterium tuberculosis, Yersinia enterocolitica and Escherichia coli. HMBPP was a 30 000-fold more potent antigen than IPP. Using mutant bacteria, we show that bacterial antigen levels for Vγ2Vδ2 T cells are controlled by MEP pathway enzymes and find no evidence for the production of 3-formyl-1-butyl pyrophosphate. Moreover, HMBPP reactivity required only germ line-encoded Vγ2Vδ2 TCR elements and is present at birth. Importantly, we show that bacterial HMBPP levels correlated with their ability to expand Vγ2Vδ2 T cells in vivo upon engraftment into severe combined immunodeficiency–beige mice. Thus, the production of HMBPP by a microbial-specific isoprenoid pathway plays a major role in determining whether bacteria will stimulate Vγ2Vδ2 T cells in vivo. This preferential stimulation by a common microbial isoprenoid metabolite allows Vγ2Vδ2 T cells to respond to a broad array of pathogens using this pathway.

Introduction

Vγ2Vδ2 T cells are a unique subset of human T lymphocytes comprising 1–4% of total adult peripheral blood T cells (1, 2). They expand during a variety of prokaryotic and eukaryotic protozoan infections such as tuberculosis (3–5), leprosy (6), typhoid fever (7), brucellosis (8), tularemia (9–11), ehrlichiosis (12), malaria (13, 14) and toxoplasmosis (15). Studies in a human peripheral blood lymphocyte-SCID mouse model (hu-PBL-SCID) demonstrated that Vγ2Vδ2 T cells help provide immunity against Escherichia coli, Morganella morganii and Staphylococcus aureus infections (16). Moreover, using rhesus monkeys, we showed that Vγ2Vδ2 T cells expand during resolution of Mycobacterium tuberculosis and Mycobacterium bovis Bacille Calmette-Guérin (BCG) infections, suggesting that γδ T cells also play a role in immunity against mycobacteria (17).

The first natural antigen structurally identified for Vγ2Vδ2 T cells was isopentenyl pyrophosphate (IPP), a metabolite all organisms use to synthesize isoprenoid compounds. Despite the presence of endogenous IPP in humans, there is no evidence that Vγ2Vδ2 T cells mediate autoimmunity, suggesting that they can distinguish between pathogen and host prenyl pyrophosphates under normal conditions (18). Two distinct pathways for IPP synthesis have been delineated that appear to contribute to this specificity (19, 20).
The mevalonate pathway is found in most eukaryotes, archaeobacteria, some eubacteria and the cytosol of plants. The second pathway, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also termed the deoxyxylulose phosphate pathway), is found in most eubacteria, apicomplexan protozoa, cyanobacteria and plant chloroplasts. In the MEP pathway, seven enzymes have been identified: Dxs, Dxr, YgbP, YchB, YgbB, GcpE and LytB (Fig. 1). A MEP pathway metabolite, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (21, 22) (also termed hydroxy-dimethylallyl pyrophosphate (HDMAPP), has been shown to have potent stimulatory activity for Vγ2Vδ2 T cells (23). Also, the in vitro stimulatory activity of E. coli could be diminished by deletion of the Dxs and GcpE enzymes in the MEP pathway (24) and increased by deletion of the LytB enzyme which is downstream from HMBPP (25). Mycoplasma species that retain MEP pathway enzymes are also able to expand Vγ2Vδ2 T cells in vitro (26). Finally, Listeria monocytogenes, that uses both pathways to make

![Fig. 1. 2-C-methyl-D-erythritol 4-phosphate (MEP) and mevalonate pathways for isoprenoid biosynthesis. The MEP pathway is found in most eubacteria, apicomplexan protozoa and chloroplasts whereas the mevalonate pathway is found in archaeobacteria, eukaryotes and the cytoplasm of plants. Genes for MEP enzymes are also termed ispC (dxr), ispD (ygbP), ispE (ychB), ispF (ygbB), ispG (gcpE) and ispH (lytB).]
isoprenoid intermediates, loses in vitro bioactivity for Vy2V62 T cells when GcpE is deleted whereas deletion of mevalonate kinase or HMG-CoA reductase did not affect bioactivity (27). Deletion of LytB in Listeria monocytogenes increases bioactivity 7-fold, a much smaller increase than is noted in E. coli (27). These findings suggest that HMBPP may act as an antigen that allows Vy2V62 T cells to distinguish exogenous from endogenous prenyl phosphate antigens.

Although HMBPP appears to be a major microbial antigen, its relationship to other described phosphoantigens, termed TUBag1, TUBag2, TUBag3 and TUBag4 (28), is unclear. The structure of TUBag1 isolated from Mycobacterium fortuitum has been reported as 3-formyl-2-butyl pyrophosphate (3-FBPP) (29). However, this compound has an identical molecular weight and chemical composition to HMBPP. The TUBag3 and TUBag4 antigens are reported to be 3-formyl-butyl conjugates to TTP and UTP (28, 30) but little is known about the presence and relative amounts of unconjugated to nucleotide-conjugated phosphoantigens in bacteria. A closely related structure, 3-formyl-2-pentyl pyrophosphate (3-FFPP) has been proposed for the second phosphoantigen, TUBag2, isolated from E. coli (31) and mycobacteria (2, 32). Also, lysates of gram-positive cocci that use the mevalonate pathway, such as Staphylococcus aureus and group A, B and C Streptococcus, stimulate Vy2V62 T cells (33, 34, data not shown) suggesting that an additional phosphoantigen (perhaps IPP) exists besides HMBPP and 3-FBPP as the major antigen for bacteria using the mevalonate pathway.

We have recently reported the synthesis of 3-FBPP (proposed as TUBag1) and find that synthetic 3-FBPP has only moderate stimulatory activity (EC50% = ~3 μM) rather than the high stimulatory activity reported (EC50% = ~5–50 nM) and that its NMR spectra does not match that reported for the natural antigen (35). Moreover, we found that the 275 Da compound in mycobacteria that was proposed to be 3-formyl-2-pentyl pyrophosphate (TUBag2) is actually 6-phosphogluconate, a compound without biological activity for Vy2V62 T cells (35). Thus, none of the TUBag antigens are 3-formyl-alkyl pyrophosphates leading to uncertainty about their structures and the relative importance of the different phosphoantigens.

To further clarify the structure and relative importance of natural phosphoantigens in different bacteria and to confirm the importance of MEP pathway enzymes in determining in vitro and in vivo stimulation of Vy2V62 T cells, we isolated bacterial antigens and identified mutations that affect bacterial antigen levels. We find that unconjugated HMBPP is the major bacterial antigen in multiple species using the MEP pathway. Consistent with this, mutations that affect bacterial antigen levels were primarily in enzymes of the MEP pathway or genes regulating this pathway. No evidence for additional enzymes that could produce 3-FBPP was found. The HMBPP metabolite was highly preferentially recognized over IPP by Vy2V62 T cells including neonatal γδ T cells. Moreover, in the human-PBL-SCID-beige mouse model, only a bacterial mutant with high levels of HMBPP expanded Vy2V62 T cells. These findings demonstrate a major role for HMBPP in determining activation of Vy2V62 T cells for bacteria using the MEP pathway.

**Methods**

**Antigens**

Ethyl pyrophosphate (EPP) was synthesized as described (1). Bromohydrin pyrophosphate was provided by Eric Oldfield (University of Illinois, Urbana-Champaign). HMBPP was synthesized as described (36).

**Derivation and maintenance of γδ T-cell clones**

T-cell clones were propagated by periodic re-stimulation as described (37). The 12G12, DG.SF68 and CP.1.15 Vy2V62 T-cell clones have been described (1). AC.2 and AC.8 are fetal liver clones (37) whereas CB.32.26 is a cord blood clone (38).

**Purification and characterization of the major antigen from Mycobacterium smegmatis and M. fortuitum**

Antigen was purified from 34 l of M. smegmatis and 4 l of M. fortuitum culture grown in Middlebrook 7H9 broth. The culture supernatants were passed through a carbon–Celite column (2) followed by tangential ultrafiltration (1000 MW cutoff, Pall-Filtron, Northborough, MA, USA). Minimal bioactivity (5–10%) was lost during these steps. Compounds in the ultrafiltrates were separated on a Q-Sepharose-Fast flow column (5–50 cm) by FPLC using an ammonium acetate gradient. Bioactive fractions were identified by their ability to stimulate the proliferation of a Vy2V62 T-cell clone and then pooled. The single peak of bioactivity was further purified by HPLC using a DEAE-5PW column (150 × 21.5 mm, Bio-Rad, Hercules, CA, USA) followed by a Mono Q column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) eluted with a triethylammonium bicarbonate gradient. The antigen was further purified using a Luna C18 column (250 × 4.6 mm, Phenomenex, Torrance, CA, USA) under ion pairing conditions with a tertiary solvent system. Solvent A: 100 mM triethylammonium bicarbonate (TEAB), pH 8.0 (prepared by bubbling CO2 through 100 mM TEA until pH 8.0); solvent B: 100 mM TEAB in 10% (v/v) methanol. The column was eluted as follows: 0–10 min isocratic in solvent A at 1 ml/min; 10–70 min linear gradient 0–10% B in A at 1 ml/min; 70–75 min linear gradient 10–50% C in A at 0.5 ml/min; 75–90 min isocratic 50% C in A at 0.5 ml/min. One minute fractions were collected from which 1 μl of each fraction was tested for stimulation of a Vy2V62 T-cell clone. Note that since this is a volatile buffer system, there is some variation in retention times for identical compounds. Each 1 min fraction was assayed for bioactivity with a γδ T-cell clone and analyzed by electrospray ionization tandem mass spectrometry in the negative mode (precursor ion and product-ion analyses) to identify and quantitate phosphate-containing compounds as previously reported (2). Electrospray ionization tandem mass spectrometry (ES MS/MS) spectra were obtained in negative ion mode using API-III, API 300 and API Qstar Pulsar I mass spectrometers (Applied Biosystems/MDS Sciex, Ontario, Canada), as described (2). The measured accurate mass of the compounds was determined by Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry using electrospray ionization in the negative ionization mode on the 7 Tesla spectrometer at...
the Environmental Molecular Sciences Laboratory. Internal calibration employing IPP and geranyl pyrophosphate was used for accurate mass measurements.

Mutation of E. coli W3110 bacteria

E. coli mutants that carry point mutations in ygbP, ychB, ygbP and gcpE were derived from E. coli W3110 following treatment with N-methyl-\textit{N}'-nitro-N-nitrosoguanidine as described (39–42). The mutant bacteria were engineered for isoprenoid metabolism through a partial mevalonate pathway by transformation of the parent bacteria with the pTMV20KM plasmid [which includes the \textit{Streptomyces} sp. strain CL190 mevalonate pathway genes, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase and isopentenyl disphosphate isomerase plus a kanamycin resistance gene (43)]. Since genes upstream of mevalonate are not included, addition of mevalonate (0.1 mg ml\textsuperscript{−1}) into the media was required for growth (Fig. 1). The DK310 LytB\textsuperscript{G120D} (pTMV20KM) strain was derived from the isopentenyl disphosphate isomerase disruptant strain, DK310, by treatment with N-methyl-\textit{N}'-nitro-N-nitrosoguanidine and transformation with pTMV20KM (44). The dxr mutant was derived by the insertion of a kanamycin resistance gene into the coding sequence of dxr as described (45) except that the parent bacteria were transformed with the pTMV20KM plasmid which includes the \textit{Streptomyces} sp. strain CL190 mevalonate pathway genes found in pTMV20 plus the HMG-CoA reductase and HMG-CoA synthase genes (43) and addition of mevalonate (0.1 mg ml\textsuperscript{−1}) into the media. For each strain, mutations were identified by plating bacteria on LB plates lacking mevalonate and culturing overnight at 37°C followed by 10 days at room temperature.

Transposon mutagenesis of the DK310 LytB\textsuperscript{G120D} mutant

Mutant strains were generated by transposon-mediated mutagenesis of the DK310 LytB\textsuperscript{G120D} (pTMV20KM) bacteria using the EZ:TNTM <DHFR-1> Tnp Transposome kit (Epitcentre, Madison, WI, USA) (44). Bacterial mutants thus generated were arrayed in a 96-well format and \( \sim \) 15 000 were screened for the loss of bioactivity with the 12G12 V\textsubscript{2}V\textsubscript{8} T-cell clone and for their ability to grow in the absence of mevalonate. To ensure the complete loss of activity, bacteria were further grown at room temperature for 4–7 days. Genomic DNA was isolated from mutants using a MasterPure\textsuperscript{TM} DNA purification kit (Epitcentre) and directly sequenced with a pair of primers specific to each end of the transposon at the University of Iowa DNA sequencing facility. The genomic transposition sites were located using BLAST programs maintained at the NCBI web site of the National Library of Medicine (http://www.ncbi.nlm.nih.gov/BLAST).

Preparing bacterial supernatants and sonicates

To test bacterial supernatants and sonicates for their ability to stimulate V\textsubscript{2}V\textsubscript{8} T cells, E. coli bacteria were grown to late stationary phase in 1 l of LB media in 2.6 l fluted Fernbach flasks by incubating for \( \sim 24 \) h at 37°C in an Innova 4400 shaker oscillating at 225 rev/min, this maximizes bioactivity. The bacteria were harvested by centrifugation at \( 380 \times g \) for 15 min at 4°C. The culture supernatant was removed and the bacteria washed twice with PBS. Bacteria from 1 l of culture were suspended in 10 ml of PBS and continuously probe sonicated for 10 min on ice at a 4.5 setting (Sonic Dismembrator Model 550, Fisher Scientific). The sonicated bacteria were centrifuged at 300 \( \times g \) for 15 min at 4°C. The supernatants from the sonicated bacteria and the culture supernatants were heated in a boiling water bath for 5 min, cooled on ice for 5 min, centrifuged at 16 000 \( \times g \) for 30 min at 4°C, filter sterilized with a 0.22-μm filter and frozen at \(-80°C\). Note that the heating caused precipitation of protein and other bacterial components that inhibit T-cell proliferation and that give falsely low estimates of bioactivity but did not affect the overall bioactivity for V\textsubscript{2}V\textsubscript{8} T cells (44). Heating also dissociates prenyl pyrophosphates from proteins and other bacterial components that prevent the passage

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Mutation</th>
<th>Mutation location</th>
<th>Growth w/o mevalonatea</th>
<th>Bioactivity (U/L)</th>
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<tbody>
<tr>
<td>—</td>
<td>W3110 dxr−</td>
<td>Insertion of a kanamycin resistance gene</td>
<td>Ball site, nucleotide 365-370</td>
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<td>18 182</td>
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<td>—</td>
<td>++++</td>
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<td>NMW33</td>
<td>Single point</td>
<td>\textsuperscript{120}L (TTG) to \textsuperscript{120}F (TTT)</td>
<td>++</td>
<td>26 087</td>
</tr>
<tr>
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<td>NMW34</td>
<td>Stop codon</td>
<td>\textsuperscript{26}Q (CAA) to Stop codon (TAA)</td>
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<td>2800</td>
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<tr>
<td>ychB</td>
<td>NMW29</td>
<td>Double point</td>
<td>\textsuperscript{112}A (GCC) to \textsuperscript{112}V (GTC) and \textsuperscript{153}A (GCC) to \textsuperscript{153}V (GTC)</td>
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<td>20 000</td>
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<tr>
<td>ygbE</td>
<td>NMW31</td>
<td>Single point</td>
<td>\textsuperscript{21}G (GGT) to \textsuperscript{21}D (GAT)</td>
<td>++</td>
<td>20 000</td>
</tr>
<tr>
<td>gcpE</td>
<td>NMW12</td>
<td>Single point</td>
<td>\textsuperscript{136}Q (GGA) to \textsuperscript{136}O (GAA)</td>
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<td>&lt;100</td>
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<tr>
<td>gcpE</td>
<td>NMW15</td>
<td>Single point</td>
<td>\textsuperscript{133}Q (CGT) to \textsuperscript{133}C (GTG)</td>
<td>–</td>
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<td>gcpE</td>
<td>NMW19</td>
<td>Single point</td>
<td>\textsuperscript{21}S (TCC) to \textsuperscript{21}F (TTC)</td>
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<td>54 545</td>
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<tr>
<td>lytB</td>
<td>LytB\textsuperscript{G120D}</td>
<td>Single point</td>
<td>\textsuperscript{120}G (GST) to \textsuperscript{120}D (GAT)</td>
<td>+</td>
<td>6 369 231</td>
</tr>
</tbody>
</table>

\(^a\)Growth was assessed in the absence of mevalonate by incubating plates overnight at 37°C and then sealing and incubating at room temperature for 10 days. Note that mutations that allow growth are associated with wild-type levels of bioactivity. Bacterial colony size $4^+ = \geq 8$ mm; $3^+ = 6–8$ mm; $2^+ = 4–6$ mm; $1^+ = 1–4$ mm. \(^b\)Growth of the dxr− strain is due to the presence of low numbers of revertants. \(^c\)LytB mutant was strain DK310 LytB\textsuperscript{G120D} (pTMV20KM).

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of prenyl pyrophosphates through membrane ultrafiltration units with molecular cutoffs greater than 1000–3000 Da.

\[ V_2^d \] T-cell proliferation assay and the quantitation of bacterial bioactivity for \[ V_2^d \] T cells

T-cell proliferation assays were performed as previously described (1). Mean proliferation and standard error of mean of triplicate cultures are shown. To quantitate bioactivity, the reciprocal dilution of the bacterial supernatant or sonicate that gave half-maximal proliferation was determined relative to a standard EPP antigen preparation (44). One unit of bioactivity was the amount of antigen in 1 ml that gave half-maximal antigen-induced proliferation of a \[ V_2^d \] T-cell clone (usually DG.SF68 or CP.1.15) and corresponds to an HMBPP concentration of 31.6 μM or 31.6 femtomoles ml⁻¹ and an IPP concentration of 3 μM or 3 nmoles ml⁻¹.

Expansion of \[ V_2^d \] T cells by non-peptide antigens

PBMC were isolated either from leukopacs or buffy coats by density centrifugation over Ficoll–Hypaque (Amersham Pharmacia Biotech). PBMCs (1 × 10⁶) were cultured in 96-well round bottom plates in complete RPMI 1640 (37) alone or in complete RPMI 1640 with 50 μM IPP or 0.316 μM HMBPP. On day 3, 100 μl of supernatant were replaced with complete medium supplemented with 1.7% human serum and 1 mM recombinant human IL-2 (Chiron Corporation, Emeryville, CA, USA). On day 7, the PBMC were harvested, counted, stained with anti-Vα2 (BB3, gift from A. Moretta) and anti-CD3 (HIT3a, BD Pharmingen, San Diego, CA, USA) monoclonal antibodies (mAbs) and analyzed by two-color flow cytometry. The Institutional Review Board at the University of Iowa approved these studies.

Expansion of \[ V_2^d \] T cells by live bacteria

E. coli wild type, LytB<sup>G120D</sup> and LytB<sup>G120D</sup> yhjK<sup>−</sup> bacteria were grown to mid-log phase and stored in LB broth containing 10% glycerol at −80°C until use. To determine colony-forming units, bacteria were washed once with PBS and grown on LB plates. For the transwell assay, 1–3 × 10⁶ bacteria were added in 0.1 ml RPMI 1640 medium to the inner wells (Corning Costar, Kennebunk, ME, USA). The inner well was separated from the outer well by a 0.4-μm membrane. PBMCs (2 × 10⁵) were added to the outer well in 0.9 ml of complete medium. After 4 h, the inner wells were removed leaving the PBMC in culture. On day 3, half of medium was replaced with complete medium supplemented with human serum and recombinant IL-2. On day 3, the PBMC were harvested, counted and \[ V_2^d \] T cells determined by flow cytometry using anti-Vα2 and anti-CD3 mAbs.

\[ V_2^d \] T-cell proliferation in human-PBL-SCID-beige mice

Homozygous C.B-Igh-1<sup>B</sup>/GbmsTac-Prrdkc<sup>GD</sup>-Lyst<sup>bg/N7</sup> (C.B-17 SCID-beige) male mice (age 5–6 weeks old) were purchased from Taconic (Germantown, NY, USA) and maintained in microisolator cages. Animals were fed autoclaved food and water and all manipulations were performed in laminar flow cabinets. In vivo expansion of γδ T cells was performed in SCID-beige mice using either HMBPP-activated or unactivated PBMC. To assess the effector capability of \[ V_2^d \] T cells, PBMC were activated for 24 h in vitro with 0.316 μM HMBPP, washed and 2.5–3 × 10⁵ cells injected i.p. into each mouse in 0.5 ml of RPMI. To assess the in vivo stimulatory capability of mutant bacteria, unactivated PBMC were used. Two hours later, each SCID-beige mouse was injected i.p. with either wild type or LytB<sup>G120D</sup> (termed lytB<sup>−</sup><sup>C</sup>) in the figures). E. coli at 1 × 10⁶–1 × 10⁷ bacteria in 0.5 ml of RPMI medium. Alternatively, varying amounts of HMBPP were given in 0.25 ml of PBS. Recombinant human IL-2 (5000 IU) (Chiron, Emeryville, CA, USA) was given i.p. every other day starting on day 0. On day 9, the mice were sacrificed and peritoneal cells were harvested by washing the peritoneum with 4 ml of PBS. The peritoneal cells were counted and analyzed by flow cytometry using anti-Vα2 and anti-CD3 monoclonal antibodies to determine the percentage of \[ V_2^d \] T cells among human CD3<sup>+</sup> T cells. The Institutional Animal Care and Use Committee of the University of Iowa approved all animal protocols. Data were tested for statistically significant differences using the non-parametric Mann–Whitney U-test.

Results

Purification of the major antigen for \[ V_2^d \] T cells from mycobacteria

Although previous studies showed that mycobacterial lysates contain non-peptide antigens that stimulate γδ T cells, there are questions about the relative importance of HMBPP, 3-FBPP and IPP as well as the relative abundance of unconjugated and nucleotide-conjugated compounds (1, 2, 28, 46–48). Therefore, we prepared lysates from various bacteria, including mycobacteria [the BCG vaccine strain of M. bovis, opportunistic (M. avium and M. fortuitum) and environmental (M. smegmatis) species], gram-positive and -negative rods and gram-positive cocci and evaluated them for their ability to stimulate \[ V_2^d \] T cells. Despite their divergent origins, all bacterial lysates stimulated \[ V_2^d \] T cells including the lysate from S. aureus, a bacterium that uses the mevalonate pathway for IPP synthesis (Fig. 2A).

To identify the compounds responsible for bioactivity in bacteria, the major peak of bioactivity was purified from M. fortuitum and M. smegmatis. Ninety to 95% of the antigenic activity in the supernatant from M. smegmatis passed through an activated charcoal–Celite column that retains nucleotide, nucleotide-conjugated and hydrophobic compounds (1). Thus, nucleotide-conjugated antigens, such as the 5‘-UTP-conjugated antigen reported for M. fortuitum (30) and the 5‘-dUTP-conjugated antigen reported for M. tuberculosis (28), accounted for, at most, 5–10% of bioactivity in M. smegmatis. Unlike antigenic activity from lysates of heat-killed M. tuberculosis (Fig. 2D), subsequent anion exchange and ion-pairing reverse phase chromatography revealed only one peak of bioactivity from both M. fortuitum and M. smegmatis. This peak of bioactivity for \[ V_2^d \] T cells on ion-pairing reverse-phase chromatography (Fig. 2B, middle panels) correlated with the presence of an ion with a mass to charge ratio (m/z) = 261 ([(M−H)<sup>−</sup>]) (Fig. 2B, upper panels). Further characterization of the m/z 261 ion
using product-ion analysis by electrospray ionization tandem mass spectrometry (ES MS/MS) revealed that the m/z 261 ion is pyrophosphorylated, as evidenced by the presence of products ions at m/z 159 (corresponding to HP$_2$O$_6^-$), m/z 97 (corresponding to H$_2$PO$_4^-$) and m/z 79 (corresponding to PO$_3^-$) (Fig. 2C). The measured accurate mass of the m/z 261 ion was 260.993655 as determined by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS).
Based on this composition of C\textsubscript{8}H\textsubscript{11}O\textsubscript{8}P\textsubscript{2} (+0.72 ppm error) (Fig. 2C). This is identical to the negative ion [M–H] of HMBPP and of 3-FBPP. Synthetic HMBPP and 3-FBPP had nearly identical major ions on collision-induced dissociation using an ion trap mass spectrometer (data not shown and 32) precluding the use of this technique to distinguish between the two compounds. Therefore, the major antigen could be either HMBPP or 3-FBPP.

The m/z 261 ion of M. smegmatis and M. fortuitum had similar retention times to TUBag1 in M. tuberculosis and the major antigen in Y. enterocolitica (Fig. 2D) and E. coli (Fig. 2E). Note, that there were minor variations in retention times for identical compounds due to the use of a volatile buffer. Nucleotide-conjugated compounds were not produced by either E. coli or Y. enterocolitica since only one peak of bioactivity was isolated (Fig. 2D and E). To determine if the level of bioactivity was related to the bacterial growth phase, cultures of M. smegmatis, M. fortuitum (data from 49) and E. coli were grown and bioactivity for V\textsubscript{2}V\textsubscript{8}T cells quantitated for different growth phases. In all three bacteria, antigen levels were highest in the late stationary phase with most of the bioactivity present in the culture supernatants (Fig. 2F). The presence of bioactivity in the culture supernatants of actively growing bacteria confirms earlier studies (30, 49) although it is not clear whether the major antigen is actively secreted or just released by dying bacteria. In some other bacterial species, antigenic activity is retained in the cytoplasm (data not shown). Since the highest levels of bioactivity are found in late stationary phase cultures, bioactivity levels for bacteria were determined at this time point.

**Mutation of genes in the MEP pathway identifies HMBPP as the primary bacterial antigen for V\textsubscript{2}V\textsubscript{8}T cells**

Given its product-ion spectra, chemical composition and the complete delineation of the MEP pathway, we hypothesized that the m/z 261 ion phosphoantigen was HMBPP rather than 3-FBPP. To test this hypothesis using a genetic approach, we made E. coli strains with mutations in enzymes of the MEP pathway, the pathway that produces HMBPP and IPP in E. coli (Table 1). Since this pathway is essential for viability, these mutants were derived from an E. coli strain that was first modified to contain a partial mevalonate pathway. The mevalonate pathway synthesizes IPP in mammals but does not make HMBPP or any other MEP pathway intermediate (Fig. 1). Mutations in MEP pathway enzymes upstream from HMBPP (YgbP, YgbB and GcpE), that completely abrogated growth in the absence of mevalonate, also markedly reduced bioactivity of V\textsubscript{2}V\textsubscript{8}T cells (Fig. 3A and Table 1). Conversely, when the downstream enzyme LytB was mutated, the bacteria showed a 300- to 1500-fold increase in V\textsubscript{2}V\textsubscript{8}T cell-bioactivity (Fig. 3A). As expected, this elevated level of bioactivity found in LytB\textsuperscript{G120D} mutant bacteria could be reduced to wild-type levels by adding fosmidomycin (FMM), a specific inhibitor of the upstream enzyme, deoxyxylulose-5-phosphate reductoisomerase (dxr). The level of HMBPP appears to be tightly regulated since bacteria with point mutations that greatly slowed but did not completely eliminate growth had similar bioactivity levels as wild-type bacteria in late stationary phase cultures (Table 1). The requirement for GcpE for biological activity confirms previous results (24, 25) and we now show that other enzymes in the pathway are similarly required.

As it is possible that 3-FBPP is produced as a side metabolite from HMBPP by a novel enzyme, we performed transposon mutagenesis of the LytB\textsuperscript{G120D} strain that accumulates high levels of bioactivity to identify genes required for bioactivity. Since transposons can insert throughout the bacterial genome, this technique should identify genes required for bioactivity for V\textsubscript{2}V\textsubscript{8}T cells potentially including genes not in the MEP pathway. Approximately 15,000 mutants were screened for their bioactivity for V\textsubscript{8}T cells and for their ability to grow independently of mevalonate (Table 2). Twenty-seven clones had lower bioactivity compared with the LytB\textsuperscript{G120D} bacteria (Fig. 3B). Direct genomic sequencing of these mutants revealed that 23 out of 27 of mutants had a transposon inserted into a known gene in the MEP pathway identifying five out of six upstream enzymes from HMBPP. These mutants did not grow in the absence of mevalonate, since they lacked the ability to synthesize IPP through the MEP pathway (Table 2 and Fig. 3B).

Two additional genes, fldA and sppA, were found to be important in the synthesis of HMBPP. fldA encodes flavodoxin I that functions as an electron donor for GcpE in the synthesis of HMBPP (21). sppA (not previously reported)
encodes a signal peptide peptidase that cleaves signal peptides and may be required for enzyme activity. Mutation of triose phosphate isomerase (tpi) also reduced bioactivity since it is required to convert dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, a precursor for the MEP pathway. Mutation of yhjK reduced bioactivity of the LytBG120D mutant but when deleted in wild-type E. coli cd bioactivity and bacterial growth were normal (K. -J. Puan and C. T. Morita, unpublished data). yhjK encodes a transmembrane signaling protein that likely regulates cyclic diguanylate monophosphate levels and that may, in turn, regulate HMBPP pool size only in the LytBG120D strain. Two mutants, cysB and yaeD, required mevalonate for growth but their HMBPP levels remained unaltered. yaeD encodes a phosphatase involved in LPS synthesis; cysB encodes a protein involved in the regulation of cysteine synthesis and may be required for activity of the mutant LytBG120D enzyme. Importantly, no enzyme that could convert HMBPP to 3-FBPP was identified. These genetic studies complement our structural analysis (Fig. 1) and functional tests on synthetic 3-FBPP (35) and suggest that HMBPP, rather than 3-FBPP, is the major antigen for bacteria using the MEP pathway.

**Fig. 3.** Bioactivity of E. coli with mutations in the MEP pathway. (A) Loss of bioactivity of E. coli with mutations in the MEP pathway correlates with mevalonate-dependent growth. Sonicates and culture supernatant were prepared from 1 l of wild-type Escherichia coli and MEP pathway-defective E. coli strains complemented with the mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase and isopentenyl disphosphate isomerase genes from the mevalonate pathway. FNM, (20 µg ml⁻¹) was included to partially block the dxr enzyme and mevalonate (1 mg ml⁻¹) was added to support isoprenoid biosynthesis through the mevalonate pathway by enzymes introduced into the bacteria. One unit of bioactivity corresponds to 31.6 femtomoles of HMBPP per milliliter. The order of the mutants (left to right) is the same as Table 1. Note that only those bacteria with complete lack of mevalonate-independent growth had low levels of bioactivity despite the presence of fosmidomycin in all mutant cultures. (B) Bioactivity of LytBG120D mutant E. coli mutated by transposons. Mutants were screened for bioactivity for Vγ2Vδ2 T cells and for mevalonate-dependent growth. Mutants are as in Table 2.

HMBPP is a highly potent phosphoantigen and its recognition can be mediated by Vγ2Vδ2 T-cell TCRs that are present at birth

To verify that HMBPP stimulates Vγ2Vδ2 T cells, synthetic HMBPP was tested for its ability to stimulate several Vγ2Vδ2 T-cell clones including fetal liver clones (AC.2 and AC.8) that
use the invariant V\textsubscript{2} (V\textsubscript{9}) chain (50); a cord blood clone, CB32.26 (38); and adult clones, 12G12, DG.SF68 and CP.1.15. For all clones, HMBPP was 30 000-fold more antigenic than IPP (Fig. 4A, half-maximal proliferation for HMBPP and IPP for the AC.2 and DG.SF68 clones was 36 pM and 1 \mu M, respectively, and unpublished data) and 100- to 300-fold more antigenic than bromohydrin pyrophosphate, a synthetic phosphoantigen. To confirm that the V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} TCR mediated HMBPP recognition, a V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} TCR transfectant, DBS43, was tested and found to release IL-2 in response to HMBPP (unpublished data). HMBPP also stimulated the expansion of V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells from normal donors (Fig. 4B). The fetal liver clones AC.2 and AC.8 use the invariant V\textsubscript{2} chain that is found in 10–30% of adult V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells (50). Reactivity to HMBPP by fetal and cord blood clones confirms ours and other’s earlier studies showing that cord blood V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells respond to HMBPP in mycobacterial lysates and to IPP, and that these responses are present at birth (38, 51–53). These results suggest that reactivity to HMBPP is a property of most V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells, including those expressing invariant V\textsubscript{2} chains.

Bacterial HMBPP levels determine the magnitude of in vitro and in vivo expansion of V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells

If HMBPP is a major determinant of V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T-cell reactivity to bacteria, we reasoned that increasing HMBPP levels would result in stronger V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T-cell responses. To determine if the levels of HMBPP in bacteria influence V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T-cell expansion in vitro, PBMC were co-cultured with live LytB\textsuperscript{G120D} that overproduce HMBPP, LytB\textsuperscript{G120D} yhj\textsuperscript{K} mutant bacteria that had extremely low bioactivity levels, and wild-type bacteria with moderate bioactivity levels in a transwell system. V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells expanded slightly more with LytB\textsuperscript{G120D} bacteria than with wild type, but much less with LytB\textsuperscript{G120D} yhj\textsuperscript{K} bacteria that had extremely low levels of bioactivity (Fig. 5).

Since V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells and prenyl pyrophosphate recognition is restricted to primates, direct testing in vivo is difficult. The hu-PBL-SCID–beige model provides a small animal model where human PBMC are transplanted into immunodeficient SCID–beige mice. Transplanted V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells have been shown to proliferate when preactivated with antigen prior to transplantation where they help provide immunity to infection with bacteria through their production of IFN-\gamma (16). Therefore, we used this model system to determine the effects of differing levels of bacterial HMBPP on the expansion of V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells in vivo.

SCID–beige mice were engrafted with HMBPP-activated PBMC (containing 1–5% V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells) and subsequently infected with bacteria. Peritoneal cells were harvested 9 days later and analyzed by flow cytometry. Mice that received HMBPP-activated PBMC, followed by either LytB\textsuperscript{G120D} \textit{E. coli} (that have very high bioactivity) or \textit{Morganella morganii}, (with more modest levels of bioactivity) showed expansion of V\textsubscript{8\textsuperscript{V}2\textsuperscript{T}} T cells that was dose dependent but not significantly different between the two bacteria (Fig. 6A and B). Similarly, wild-type and LytB\textsuperscript{G120D} bacteria elicited roughly similar levels of V\textsuperscript{8\textsuperscript{V}2\textsuperscript{T}} T-cell expansion. This result is consistent with previous in vitro studies showing that LPS alone could stimulate antigen-activated V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells to expand and secrete IFN-\gamma (54). These findings suggest that after non-peptide antigen stimulation in vitro, subsequent in vivo V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T-cell expansion is less dependent on antigen levels.

In contrast, when unactivated PBMC were used for engraftment, significantly higher levels of V\textsubscript{8\textsuperscript{V}2\textsuperscript{T}} T-cell expansion were found only with infection by LytB\textsuperscript{G120D} \textit{E. coli} as compared with wild-type \textit{E. coli} or non-infected controls (Fig. 6D, right). V\textsubscript{8\textsuperscript{V}2\textsuperscript{T}} T-cell expansion was also dependent on bacterial numbers, as shown in an independent experiment with a different donor (Fig. 6D, left). These expansions occurred in the absence of exogenously added IL-2. Thus, bacterial HMBPP levels likely play an important role in determining in vivo responses by V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells.

To determine if HMBPP could directly stimulate V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells in vivo, unactivated PBMC were transplanted into SCID–beige mice and then stimulated with synthetic HMBPP. HMBPP stimulated the expansion of resting V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T cells such that their absolute numbers and percentage of CD3 T cells were significantly increased (Fig. 7). Unlike expansions with bacterial infection, this expansion was dependent on exogenous IL-2 (data not shown).

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### Table 2. Transposon mutants of DK310 LytB\textsuperscript{G120D} \textit{Escherichia coli} bacteria

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of isolates</th>
<th>Product</th>
<th>Fold reduction in V\textsubscript{2\textsuperscript{V}2\textsuperscript{T}} T-cell bioactivity</th>
<th>Mevalonate required for growth</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>tpi</td>
<td>1</td>
<td>triose phosphate isomerase</td>
<td>8</td>
<td>No</td>
<td>Isomerizes dihydroxy-acetone-phosphate to glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>dks</td>
<td>14</td>
<td>Deoxoxyxyllose synthase</td>
<td>1183</td>
<td>Yes</td>
<td>Synthesizes deoxoxyxyllose-5-phosphate</td>
</tr>
<tr>
<td>dxt</td>
<td>1</td>
<td>Deoxoxyxyllose reductoisomerase</td>
<td>1650</td>
<td>Yes</td>
<td>Synthesizes ME4P</td>
</tr>
<tr>
<td>ychB</td>
<td>2</td>
<td>CDP-ME kinase</td>
<td>6822</td>
<td>Yes</td>
<td>Synthesizes CDP-ME2P</td>
</tr>
<tr>
<td>ygbB</td>
<td>2</td>
<td>MECDP synthase</td>
<td>1650</td>
<td>Yes</td>
<td>Synthesizes ME-2,4cPP</td>
</tr>
<tr>
<td>gcpE</td>
<td>4</td>
<td>HMBPP synthase</td>
<td>7226</td>
<td>Yes</td>
<td>Synthesizes HMBPP</td>
</tr>
<tr>
<td>fdA</td>
<td>1</td>
<td>Flavodoxin A</td>
<td>374</td>
<td>Yes</td>
<td>Electron transferase</td>
</tr>
<tr>
<td>spA</td>
<td>1</td>
<td>Signal peptide protease A</td>
<td>220</td>
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<tr>
<td>yhjK</td>
<td>1</td>
<td>Transmembrane receptor</td>
<td>24 220</td>
<td>No</td>
<td>Regulates c-di-GMP levels</td>
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<tr>
<td>yeaD</td>
<td>1</td>
<td>Phosphatase</td>
<td>1</td>
<td>Yes</td>
<td>Cell wall synthesis</td>
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<tr>
<td>ysbB</td>
<td>1</td>
<td>Transcription factor</td>
<td>0</td>
<td>Yes</td>
<td>Regulates cysteine synthesis</td>
</tr>
</tbody>
</table>

c-di-GMP, cyclic diguanylate guanosine monophosphate; CDP-ME, 4-diphosphocytidyl-2,4-dimethyl-D-erythritol; MECDP, 2,4-dimethyl-D-erythritol 2,4-cyclodiphosphate; ME-2,4cPP, 2,4-dimethyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate.
Discussion
In this study, we show that the level of HMBPP in bacteria is a major factor in determining in vivo responses in the hu-PBL-SCID-beige mouse model by V\textsubscript{\gamma}\textdelta T cells. We find that HMBPP is the primary antigen for V\textsubscript{\gamma}\textdelta T cells in mycobacteria and in the gram-negative rods, Escherichia coli and Yersinia enterocolitica. We confirm and extend previous studies by showing that mutations in all six enzymes upstream of HMBPP in the MEP pathway abolished or greatly diminished bioactivity, whereas mutation of the downstream LytB enzyme greatly increased bioactivity. Infection with the LytBG\textsubscript{120D} mutant also expanded V\textsubscript{\gamma}\textdelta T cells in the hu-PBL-SCID-beige mouse model. The magnitude of the V\textsubscript{\gamma}\textdelta T-cell expansion was related to the HMBPP levels in the bacteria, and synthetic HMBPP was highly active on a molar basis in stimulating V\textsubscript{\gamma}\textdelta T cells both in vitro and in vivo. Since the MEP pathway is widely distributed in many important human pathogens including Mycobacteria, gram-negative bacteria and apicomplexan protozoa, recognition by V\textsubscript{\gamma}\textdelta T cells of a metabolite in this pathway allows V\textsubscript{\gamma}\textdelta T cells to combat infection by a broad range of microbial pathogens.

Our study also helps to address the question of the structure of the 262 Dalton phosphoantigen (TUBag1). On transposon mutagenesis of the LytBG\textsubscript{120D} E. coli mutant, no genes were identified that could encode an enzyme that would produce 3-FBPP. Moreover, our studies on synthetic 3-FBPP (35) show that this compound has only low to moderate activity for V\textsubscript{\gamma}\textdelta T cells rather than the high activity reported for TUBag1 (29) and has a different NMR spectra than that reported for TUBag1 (29). Similarly, the 275 Dalton compound from
mycobacteria that was proposed as 3-formyl-pentyl pyrophosphate is actually 6-phosphogluconate, a biologically inactive compound (35). Nucleotide conjugated forms of TUBag1 do not contribute significant amounts of bioactivity in both gram-negative rods and rapid growing mycobacteria. Also, none of the other metabolites in the MEP pathway have significant bioactivity for Vγ2Vδ2 T cells (31). Taken together and with other reported genetic studies and structural studies on phosphoantigens (23, 24, 26, 27, 55, 56), we conclude that HMBPP is most likely the 262 Dalton antigen isolated from mycobacteria and from gram-negative rods that use the MEP pathway.

Using transposon and chemical mutagenesis, we have identified all of the genes of enzymes in the MEP pathway as affecting bioactivity levels for Vγ2Vδ2 T cells. Mutations in any of these genes blocked bacterial growth on media without mevalonate, and decreased bioactivity of the LytBG120D mutant to wild-type levels. We also found that the fldA gene, encoding flavodoxin I, was essential both for the MEP pathway and for bioactivity. Flavodoxin contains a flavin mononucleotide and donates electrons to a number of iron-containing proteins (57, 58). One protein that likely requires flavodoxin activity is GcpE, a [4Fe-4S] protein that, via two one-electron transfers, catalyses the synthesis of HMBPP (59). After disrupting the fldA gene, HMBPP was not produced and bacteria stopped growing; complementation of mutants with flavodoxin restored growth (44). LytB is also a [4Fe-4S] protein (60) and flavodoxin may be required for its enzymatic activity. Although the mutants had lower bioactivity for Vγ2Vδ2 T cells, the ability of mutant bacteria to stimulate Vγ2Vδ2 T cells was not completely abolished. This bioactivity is probably due to either HMBPP produced by residual MEP enzyme activity or to IPP. Consistent with this latter hypothesis, eubacteria that use the mevalonate pathway, such as Staphylococcus and Streptococcus, also contain a phosphoantigen that is likely to be IPP (unpublished data, 33, 34).

The Vγ2Vδ2 TCR mediates recognition of HMBPP (61) and Vγ2Vδ2 T cells do not require antigenic selection to enrich for rare reactive clones. We previously showed that in cord blood, Vγ2Vδ2 T cells expand to high numbers when cultured with M. tuberculosis lysates (51) and that cord blood Vγ2Vδ2 T-cell clones isolated without antigenic stimulation respond to non-peptide antigens (38). Here, we demonstrate that fetal liver clones and a cord blood clone respond to HMBPP like adult Vγ2Vδ2 T cells. This strong reactivity for HMBPP is found in many cord blood and fetal Vγ2Vδ2 clones (38) including those carrying the germ line-encoded invariant Vγ2 gene sequence (such as AC.2 and AC.8) (50). This invariant Vγ2 junctional sequence is commonly expressed by Vγ2Vδ2 T cells since it was found in 11–30% of Vγ2Jγ1.2 rearrangements from nine children (50), in 10–17.6% of Vγ2Jy1.2 rearrangements from five adults (62) and in 6.5% of functional Vγ2Jy1.2 rearrangements before and 11.9% after IPP stimulation of one donor (63). There is also likely to be selection for more reactive Vγ2Vδ2 T cells during infancy as evidenced by the predominance of Vγ2Vδ2 T cells expressing Vγ2 chains using the Jγ1.2 region and with a hydrophobic residue in the CDR3β region that are not commonly seen in fetal Vγ2Vδ2 T cells (64).

A recent estimate of precursor frequency of naive CD8 T cells specific for the H-2Db-restricted GP33-41 epitope of lymphocytic choriomeningitis virus was one in 200,000 (65), whereas Vγ2Vδ2 T cells constitute one in 618 T cells in cord blood (38) and one in 25–100 T cells in adults (1, 66, 67). Since most Vγ2Vδ2 T-cell clones isolated from adults by sorting and lectin stimulation respond to mycobacterial lysates [10 reactive/10 clones, C. T. Morita unpublished observation, 11/14 clones (68), and 25/26 clones (67) for 46/50 clones (92%)], it is likely that the majority of adult Vγ2Vδ2 T cells respond to HMBPP. Thus, unlike αβ T cells specific for peptides, a previous encounter with a specific bacteria is not required to amplify adult HMBPP-specific Vγ2Vδ2 T cells since earlier infections or exposure to endogenous IPP has amplified further the already high percentage of reactive Vγ2Vδ2 T cells (69). This ability of Vγ2Vδ2 T cells to recognize HMBPP may be vital in containing infections prior to the onset of adaptive αβ T-cell and B-cell responses.

Since murine γδ T cells do not respond to prenyl pyrophosphate antigen, the hu-PBL-SCID-beige mouse model offers a small animal model to study human Vγ2Vδ2 T-cell functions in vivo. Previous studies using the hu-PBL-SCID model frequently relied on the prior activation of PBMC in vitro with an agonistic anti-CD3 antibody (70). In another study, Vγ2Vδ2 T cells were activated in vitro with the alkylamine, isobutylamine, prior to transfer to generate Vγ2Vδ2 T-cell responses in vivo (16). Activating PBMC with HMBPP in vitro increased the responsiveness of the Vγ2Vδ2 T cells to subsequent infection with different E. coli bacteria. This is analogous to CD8+ αβ T cells where initial priming with antigen ex vivo sensitizes them for greater proliferation and differentiation (71, 72). In contrast, the elevated levels of HMBPP found with mutant LytBG120D E. coli stimulated Vγ2Vδ2 T-cell expansion in SCID-beige mice engulfed without requiring preactivation with antigen or exogenously added IL-2. Similarly, synthetic HMBPP was able to expand transferred Vγ2Vδ2 T cells in hu-PBL-SCID-beige mice but this expansion required exogenously added IL-2 similar to the

Fig. 5. Bacterial HMBPP levels determine in vitro expansion of Vγ2Vδ2 T cells. Live wild-type or mutant bacteria (1 or 3 × 10⁶) were added to the inner well of a transwell where they were separated from PBMC in the outer well by a 0.4-μm membrane. After 4 h, the inner wells were removed. On day 6, PBMC were harvested, counted and Vγ2Vδ2 T cells determined by flow cytometry using anti-Vδ2 and anti-CD3 mAbs. Data shown are from one donor and are representative of results with seven donors.

Microbial metabolite recognition by γδ T cells
Fig. 6. Bacterial HMBPP levels determine in vivo expansion of unactivated Vγ2Vδ2 T cells in the hu-PBL-SCID–beige mouse model. (A, B) *Escherichia coli* LytB<sup>120D</sup> or *Morganella morganii* expand HMBPP-activated Vγ2Vδ2 T cells in a dose-dependent manner. SCID–beige mice were reconstituted with 3 x 10<sup>7</sup> HMBPP-activated PBMC i.p. and subsequently challenged with increasing numbers of *E. coli* LytB<sup>120D</sup> or *M. morganii* bacteria. After 9 days, cells were harvested and Vγ2Vδ2 T cells determined by two-color flow cytometry. (A) Two-color flow cytometric analysis of representative mice with or without bacteria. (B) Bacterial dose response of HMBPP-activated Vγ2Vδ2 T cells. Total Vγ2Vδ2 T cells were up to 3-fold greater in mice receiving bacteria. (C) Expansion of HMBPP-activated Vγ2Vδ2 T cells by both wild-type and LytB<sup>120D</sup> *E. coli*. SCID–beige mice were reconstituted with HMBPP-activated PBMC as in (A). Note the similar increases in Vδ2<sup>+</sup> T cells in mice receiving wild-type bacteria and LytB<sup>120D</sup> bacteria that have elevated levels of HMBPP. (D) Expansion of unactivated Vγ2Vδ2 T cells is dependent on HMBPP levels. SCID–beige mice were reconstituted with unactivated PBMC followed by infection with either wild-type or LytB<sup>120D</sup> bacteria. Left and right panels represent two independent experiments with three mice per group and 10 mice per group, respectively. *P < 0.01. Note that only mice receiving the LytB<sup>120D</sup> bacteria showed expansion of Vγ2Vδ2 T cells.
system (αβ T cells and follicular B cells) is naive. This ability parallels that of marginal zone B cells that are programmed to mount rapid and intense antibody responses to blood-borne pathogens (77, 78). Similar to γδ T cells, some marginal zone B cells use their invariant or V_{HV}-restricted antibody receptors to recognize non-peptide antigens found in both pathogens and self. But for some marginal zone B cells the targets are phosphorylcholine (phospholipids) or polysaccharide compounds (79).

The ability of γδ T cells to preferentially recognize a foreign metabolite is also reminiscent of pattern recognition by Toll-like receptors (TLRs) of the innate immune system. Each TLR recognizes conserved structures produced by or in response to different microbes (80). Moreover, like Vγ2Vδ2 TCR recognition of endogenous IPP, some TLRs, such as TLR9, also recognize endogenous DNA under certain conditions (81). The microbial TLR ligands are abundant, distributed in a wide array of microorganisms, and predominantly non-peptidic. Similarly, HMBPP is present in a wide array of both prokaryotic and eukaryotic microorganisms that use the MEP pathway. Vγ2Vδ2 T cells also express TLR2 and the recognition of non-peptide antigens is enhanced by the presence of TLR ligands either directly (82) or indirectly through their stimulation of IFN-ω/β from antigen-presenting cells (83, 84).

Vγ2Vδ2 T cells may be particularly important in immunity to infections caused by intracellular bacteria or protozoa that subvert the innate and adaptive immune systems. Many of the infections that expand Vγ2Vδ2 T cells are by intracellular microbes [reviewed in (85, 86)] and the expansion of Vγ2Vδ2 T cells correlated with clearance of mycobacteria in rhesus monkeys (17). In the hu-PBL-SCID mouse model, Vγ2Vδ2 T cells also help to protect mice from infections with E. coli, S. aureus and M. morganii by the production IFN-γ and other cytokines (16). Vγ2Vδ2 T cells can recognize cells infected with M. tuberculosis, M. bovis BCG and Salmonella typhimurium (7, 87–89) and kill the infected cells through perforin-and Fas ligand-dependent pathways (90–93). Released bacteria and malarial parasites can then be killed by granulysin (88, 90, 93–97). Activated Vγ2Vδ2 T cells secrete a variety of cytokines and chemokines [chemokine production is reviewed in (98)]. Most Vγ2Vδ2 T cells secrete IFN-ω, tumor necrosis factor-α and other inflammatory cytokines (37, 99). They also secrete inflammatory chemokines such as MIP-1α (CCL3), MIP-1β (CCL4), lymphotactin (XCL1) and RANTES (CCL5) (100–102). Vγ2Vδ2 T cells can also kill bacteria by secreting the cathelicidin, LL-37, which has an anti-bacterial effect on Brucella suis (103). Besides their direct role in microbial immunity, Vγ2Vδ2 T cells may also be important for the maintenance of tissue integrity and to speed tissue repair through the production of connective tissue growth factors (104, 105) and metalloproteinases (106). They may also serve to regulate αβ T cell and innate immune responses as has been shown in mice [reviewed in (107, 108)].

Besides responding to HMBPP, Vγ2Vδ2 T cells also recognize the endogenous IPP metabolite when overproduced by certain tumor cells (109) or by pharmacological inhibition of farnesyl pyrophosphate synthase by bisphosphonates or alkylamines (109–111). This overproduction of IPP appears to determine Vγ2Vδ2 T-cell recognition of some B-cell tumors.
Microbial metabolite recognition by γδ T cells

(109). Vδ2Vε2 T cells also recognize and kill a wide variety of tumor cells including prostate carcinomas, renal cell carcinomas, nasopharyngeal carcinomas and colon carcinomas probably through non-TCR mediated, NK receptor recognition (112–116). Since immunotherapy with Vδ2Vε2 T cells can control B cell malignancies (75), Vδ2Vε2 T cells may naturally perform tumor surveillance and could be used for immunotherapy of a number of different cancers.

In summary, the preferential recognition of the exogenous isoprenoid metabolite, HMBPP, over endogenous isoprenoids is likely to play a central role in the immune function of Vδ2Vε2 T cells and parallels antigen recognition by adaptive marginal zone B cells and pattern recognition by innate cells. Exploiting this unique property of Vδ2Vε2 T cells may result in new vaccines for bacterial infections and new immunotherapies for malignancies.

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Abbreviations

EPP  ethyl pyrophosphate
ES MS/MS  electrospray ionization tandem mass spectrometry
3-FBP  3-formyl-1-butyl pyrophosphate
FT-ICR  Fourier transform ion cyclotron resonance
HDMAPP  hydroxy-dimethylallyl pyrophosphate
HMPP  (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
hu-PBL-SCID  human peripheral blood lymphocyte-SCID mouse model
IPP  isopentenyl pyrophosphate
MEP  2-C-methyl-D-erythritol-4-phosphate
TEAB  triethylammonium bicarbonate
TLR  Toll-like receptor

References


