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Metabolic Engineering of *Salmonella* Vaccine Bacteria To Boost Human Vγ2Vδ2 T Cell Immunity

Grefachew Workalemahu,∗† Hong Wang,∗† Kia-Joo Puan,∗† Mohanad H. Nada,∗†‡§ Tomohisa Kuzuyama,‡ Bradley D. Jones,‡#*** Chenggang Jin,∗†,‡ and Craig T. Morita∗†,‡§

Human Vγ2Vδ2 T cells monitor isoprenoid metabolism by recognizing foreign (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a metabolite in the 2-C-methyl-D-erythritol-4-phosphate pathway used by most eubacteria and apicomplexan parasites, and self isopentenyl pyrophosphate, a metabolite in the mevalonate pathway used by humans. Whereas microbial infections elicit prolonged expansion of memory Vγ2Vδ2 T cells, immunization with prenyl pyrophosphates or aminobisphosphonates elicit short-term Vγ2Vδ2 expansion with rapid anergy and deletion upon subsequent immunizations. We hypothesized that a live, attenuated bacterial vaccine that overproduces HMBPP would elicit long-lasting Vγ2Vδ2 T cell immunity by mimicking a natural infection. Therefore, we metabolically engineered the avirulent *ara−* *Salmonella enterica* serovar Typhimurium SL7207 strain by deleting the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding mevalonate pathway enzymes. *LytB−* *Salmonella* SL7207 had high HMBPP levels, infected human cells as efficiently as did the wild-type bacteria, and stimulated large ex vivo expansions of Vγ2Vδ2 T cells from human donors. Importantly, vaccination of a rhesus monkey with live *lytB−* *Salmonella* SL7207 stimulated a prolonged expansion of Vγ2Vδ2 T cells without significant side effects or anergy induction. These studies provide proof-of-principle that metabolic engineering can be used to derive live bacterial vaccines that boost Vγ2Vδ2 T cell immunity. Similar engineering of metabolic pathways to produce lipid Ags or B vitamin metabolite Ags could be used to derive live bacterial vaccine for other unconventional T cells that recognize nonpeptide Ags.


Unconventional, innate-like T cells, such as γδ T cells, αβ invariant NKT (iNKT) cells, and mucosal-associated invariant αβ T cells, have unique roles that bridge innate and adaptive immunity by responding to nonpeptide stim-

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Abbreviations used in this article: BCG, bacillus Calmette–Guerin; BrHPP, bromo-hydrin pyrophosphate; DMAPP, dimethylallyl pyrophosphate (diphosphate); HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (diphosphate); HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; iNKT, invariant NKT; IPP, isopentenyl pyrophosphate (diphosphate); kDa, kilodalton resistance gene; LB, Luria–Bertani; MEP, 2-C-methyl-D-erythritol-4-phosphate; MOI, multiplicity of infection; OD600, OD at 600 nm;
ized SCID mouse models (20–23). In clinical trials, synthetic vaccines (bromohydrin pyrophosphate or aminobisphosphonates with IL-2) that stimulate Vγ2Vδ2 T cells resulted in complete or partial remissions for some patients with lymphoma (24) and stabilized disease in patients with metastatic prostate cancer (25) without major toxicities or autoimmunity. However, although initially effective, these vaccines result in anergy and deletion of Vγ2Vδ2 T cells after a few immunizations (26–28). In contrast, Vγ2Vδ2 T cell responsiveness is preserved after mycobacterial (29) or listeria (30) infections, with reinfection resulting in earlier and more vigorous Vγ2Vδ2 T cell expansion.

Metabolic engineering of bacteria has focused on altering bacteria for drug or chemical synthesis or for the generation of alternative fuels (31–33). Directed changes in bacterial metabolism are made by modifying specific biochemical reactions or by introducing new ones to improve pathway efficiency or to produce new compounds. We reasoned that metabolic engineering could be used to develop a bacterial vaccine that overproduces HMBPP to stimulate for Vγ2Vδ2 T cells and that avoids the anergy and deletion that can occur with direct immunization with prenyl pyrophosphates or aminobisphosphonates. The bacteria would provide the necessary adjuvants to activate innate immunity and Ags to stimulate CD4+ T cells to provide help to Vγ2Vδ2 T cells.

Attenuated Salmonella bacteria have been used as live vaccines to prevent human typhoid fever caused by systemic infection with Salmonella enterica serovar Typhi (34, 35), fowl typhoid caused by S. enterica serovar Gallinarum, and infection of egg-laying hens by S. enterica serovars Typhimurium and Enteritidis. However, the vaccines for human typhoid fever are only 50–80% protective (36) and there are no vaccines to prevent human nontyphoidal salmonellosis typically caused by S. enterica serovars Typhimurium and Enteritidis. Nontyphoidal salmonellosis is a major cause of bacterial gastroenteritis in both developed and underdeveloped nations and was estimated to have caused 93.8 million cases and 155,000 deaths worldwide in 2006 (37). Although generally restricted to the gastrointestinal tract, in AIDS patients and other immunocompromised patients these infections can be invasive, resulting in bacteraemia and in death in 20–25% of African cases (38, 39). Thus, development of Salmonella vaccines targeting the Typhimurium serovar could help prevent these deaths.

In this study, we provide proof-of-principle that metabolic engineering can be used to develop bacterial vaccines by engineering a vaccine strain of S. enterica serovar Typhimurium to overproduce HMBPP, the major Vγ2Vδ2 T cell stimulator. The engineered bacteria expand human Vγ2Vδ2 T cells from PBMCs upon ex vivo culture and monkey Vγ2Vδ2 T cells upon in vivo immunization.

Materials and Methods
Bacterial strains and plasmids

The attenuated vaccine strain araO– S. enterica serovar Typhimurium SL7207 (also termed 23377-65; hisG46 DELA07 (aro54::Tn10, Tec-s) was used for this study. SL7207 was derived from strain SL2361 by extensive deletion of the araO gene for 6-oxopropylyshikimate-3-phosphate synthase in aromatic amino acid and folic acid biosynthesis, which renders the bacteria auxotrophic for para-aminobenzoic acid and 2,3 dihydroxybenzoic acid, two compounds that are not normally produced by mammals. To complement for the loss of the MEP pathway in this bacteria, the mevalonate pathway gene cluster from Streptomyces sp. strain CL190 was used (40, 41). This cluster contains all six genes for the mevalonate pathway enzymes (3-hydroxy-3 methylglutarly-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA synthase, isopentenyl pyrophosphate isomerase, mevalonate kinase, mevalonate pyrophosphate decarboxylase, and phosphomevalonate kinase) required for the production of IPP and dimethylallyl pyrophosphate (DMAPP). The high-copy-number pTMV19 kanamycin resistance gene (kmr) plasmid was derived by inserting the Streptomyces gene cluster from the pUMV19 plasmid (41) into the cloning site of the pTQt18 plasmid and a kanamycin resistance gene into the ampicillin resistance gene. This is a high-copy-number plasmid based on a pUC plasmid (42). The low-copy-number pMMV19 kmr plasmid was derived by inserting the Streptomyces gene cluster into the cloning site and a kanamycin resistance gene into the ampicillin resistance gene of the pMW118 plasmid. This is a low-copy-number plasmid (fewer than five copies per bacteria) based on the pSC101 plasmid (43).

Metabolic engineering of araO– S. enterica serovar Typhimurium SL7207 by deletion of the lytB gene and complementation by mevalonate pathway genes

The lytB gene was deleted from araO– S. enterica serovar Typhimurium Typhimurium SL7207 by homologous recombination using the “one-step inactivation” technique with the λ Red system (44). As detailed in Supplemental Fig. 1, araO– Salmonella was transformed by electroporation (2.5 kV, 25 μF, 200 ohms; Gene Pulser II with Pulse Controller Plus from Bio-Rad, Hercules, CA) with either the pTMV19 kmr or the pMMV19 kmr plasmid containing the genes for mevalonate pathway enzymes. Plasmid retention was selected for with kanamycin (25 μg/ml). Next, the temperature-sensitive pKD46 plasmid containing the λ Red locus was introduced into the bacteria and plasmid retention was selected for with ampicillin (100 μg/ml) and incubation at 30°C. The resulting transformants were then grown for 2 d at 30°C with fosmidomycin (12.5 μg/ml) to block the MEP pathway. This switches isoprenoid synthesis to the introduced mevalonate pathway and greatly improves recovery of deletion mutants. On the third day, the bacteria were diluted to 0.1 OD at 600 nm (OD600) and grown with fosmidomycin and 1-aminobutyric acid (200 μM) (to inhibit the mevalonate enzymes) until reaching an OD600 of 0.4–0.6, at which time they were either used for electroporation or frozen. A PCR product targeting the lytB gene was produced by using primers composed of sequences flanking the lytB gene (50 bp) and sequences from the pKD3 chloramphenicol resistance gene (21 bp). The sequence of the lytB forward primer was 5′-CATCTTGATATTTGGAATCAGCCGCTGGAGGATCATAACAGTTGGTCAACGTAGCGTCATCAGGCAGATTTCTGAAATTGGTGAATCTAGATGTTAGC-3′ and the lytB reverse primer was 5′-TAAAGATCCTGTAACAGTGGCTCATGACGCAGATTTCTGAAATTTGGAATCAGCCGCTGGAGGATCTATAACAGTTGGTCAACGTAGCGTCATCAGGCAGATTTCTGAAATTGGTGAATCTAGATGTTAGC-3′. The resulting 1.1-kb DNA fragment was introduced by electroporation into araO– Salmonella containing pTMV19 kmr or pMMV19 kmr and pKD46. After incubation for 2 h, deletion mutants were selected by spreading on Luria–Bertani (LB) agar plates containing chloramphenicol (10–25 μg/ml) and incubating at 30°C. Chloramphenicol-resistant deletion mutants were then grown at 37°C to eliminate the temperature-sensitive pKD46 plasmid.

Preparation of bacterial sonicates and supernatants and assaying the bioactivity of Salmonella bacteria for human Vγ2Vδ2 T cells

To quantitate the bioactivity of Salmonella bacterial mutants for Vγ2Vδ2 T cells, bacterial clones were grown in 100 ml LB media in baffled 500-ml Erlenmeyer flasks to late stationary phase (~24 h) at 37°C in an Innova 4400 shaker (New Brunswick Scientific, Einfelt, CT) oscillating at 225 revolutions/min. Min. Bacteria were harvested and washed twice with Milli-Q H2O. The bacteria were then suspended in 10 ml Milli-Q H2O and continuously probe sonicated for 10 min on ice at the 4.5 setting using a microtip probe (Q700 sonicator; Qsonica, Newtown, CT). The bacterial sonicates and culture supernatants were heated in a boiling water bath for 5 min, cooled on ice for 5 min, centrifuged at 16,000 × g for 30 min at 4°C, filter sterilized with a 0.22-μm filter, and, in some cases, size fractionated by ultrafiltration (3000 Da cut-off units; Pall, Port Washington, NY) for the pass-through fraction. The samples were stored at −80°C until testing. To assay bioactivity, 5–10× 10^5 12G12 Vγ2Vδ2 T cells were cultured with 5–10× 10^5 mitomycin C-treated Va-2 cells (a transformed human fibrosarcoma cell line) as APCs in 200 μl complete medium (RPMI 1640 with 8% FBS, l-glutamine, sodium pyruvate, nonessential MEM amino acids, essential MEM amino acids, HEPES, and 2-ME) in round-bottom 96-well plates. Culture supernatants and bacterial sonicates were added to the cultures at half-log dilutions and incubated at 37°C. The cultures were pulsed with 1 μCi [H]thymidine after 24 h and harvested 16–24 h later (45). To quantitate bioactivity, the dilution of the bacterial supernatant or sonicate that stimulated half-maximal proliferation was determined and the reciprocal of this dilution gave the units of bioactivity (45). To control for variations in this assay (~3-fold), monoethyl pyrophosphate (mEPP) and HMBPP were used as positive controls to standardize the cultures at half-log dilutions and incubated at 37°C. One unit per milliliter of bioactivity corresponds to an HMBPP concentration of 31.6 pM (31.6 fmol/ml) or an IPP concentration of 3 μM (3 mmol/l).

Growth and morphology of Salmonella vaccine bacteria

Wild-type araO– S. enterica serovar Typhimurium SL7207 bacteria and lytB deletion mutants were cultured overnight and then diluted to 0.05
OD\textsubscript{600} in 100 mL LB broth and cultured for 96 h with periodic measurement of absorbance. To assess morphology, bacteria were grown overnight on LB plates at 37°C. Then a colony was resuspended in H\textsubscript{2}O\textsubscript{2}, spread on a slide, and Gram-stained (BD Biosciences, Franklin Lakes, NJ).

**Invasiveness and intracellular survival of Salmonella bacteria in epithelial cells**

To assess the ability of Salmonella bacterial mutants to invade and survive in human epithelial cells, Salmonella bacteria were cultured with HeLa tumor cells (derived from cervical tissue and positive for keratin) and intracellular bacterial numbers determined at 2 and 24 h postinfection in a standard gentamicin survival assay (46). For this assay, HeLa cells were grown in 24-well plates for 1 d prior to infection. Salmonella bacteria that had been grown to midlog exponential phase were added at a 1:10 multiplicity of infection (MOI). For the 2-h assay, the cells were washed at 2 h and intracellular bacteria recovered by lysing the cells with 0.1% Triton X-100. For the 24-h assay, the cells were washed with PBS 2 h postinfection and incubated in media supplemented with high-dose gentamicin (100 \textmu g/mL) for an additional 2 h, washed again, and further incubated for 20 h with media supplemented with low-dose gentamicin (25 \textmu g/mL) to kill any remaining extracellular bacteria. At 24 h postinfection, the cells were lysed as above. Intracellular bacterial numbers were assessed by plating the cell lysate on LB agar plates to determine CFUs. The 2-h incubation period assessed bacterial invasiveness whereas the 24-h incubation period assesses bacterial intracellular survival.

**Ex vivo expansion of human V\textsubscript{y}V\textsubscript{2}V\textsubscript{6}V\textsubscript{2} T cells stimulated by live Salmonella bacteria**

For ex vivo expansion of V\textsubscript{y}V\textsubscript{2}V\textsubscript{6}V\textsubscript{2} T cells stimulated by live Salmonella bacteria, PBMCs were isolated from the blood or leukocytes of normal human donors by Ficoll-Hypaque density centrifugation. PBMCs (1 \times 10\textsuperscript{5}) in 0.2 ml supplemented RPMI 1640 media (termed P-media) (47) were added to 96-well round-bottom wells and infected with Salmonella bacteria at an MOI of 1:1. After 6 h of infection, the cells were washed, treated with high-dose gentamicin (100 \textmu g/mL) for 2 h, followed by low-dose gentamicin (25 \textmu g/mL) for the remainder of the culture period. The cells were then incubated at 37°C in 5% CO\textsubscript{2} at 37°C for 3 d. 2 \textmu m FITC-anti-CD3, PE-anti-CD8, and V\textsubscript{y}V\textsubscript{6} IL-2 was added and the cells were harvested on day 9. For the Transwell assay, 1-3 x 10\textsuperscript{5} bacteria were added in 0.1 ml RPMI 1640 medium to the inner wells of a Transwell plate (Corning Costar, Kenebunk, ME). The inner well was separated from the outer well by a 0.4-\mu m membrane. PBMCs (2 \times 10\textsuperscript{5}) were added to the outer well in 0.9 ml medium. After 4 h, the inner wells were removed. On day 3, 2 nM IL-2 was added and the media were changed every 3 d. On day 9, the cells were harvested, washed, and V\textsubscript{y} and V\textsubscript{2}V\textsubscript{6}V\textsubscript{2} T cells enumerated by flow cytometric analysis using an LSR II flow cytometer and CellQuest or FACSDiva software (BD Biosciences, Franklin Lakes, NJ).

**Immunization of rhesus monkeys with live Salmonella bacteria**

Female rhesus monkeys of Chinese origin were obtained from the National Institutes of Health specific pathogen-free breeding colony (Corpus Christi, TX) for use in these studies (Covance Research Products, Denver, PA). The monkeys were free of infection with retroviruses, herpes B virus, and Shigella. Serum and urine samples were collected from all animals, and all animals were maintained and used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Iowa City Veterans Health Care System. To prepare Salmonella vaccines for immunization, the bacteria were cultured in LB media overnight, diluted the next day, and grown to midlog phase. The bacteria were then harvested, washed twice with PBS, and resuspended in PBS to give the desired concentration of bacteria based on the absorbance at 600 nm. The bacterial dose given was verified by determining CFUs in the bacterial suspensions. For intranasal immunization of monkeys, the monkeys were anesthetized and 50–100 \textmu l bacterial suspension instilled in each nostril. The intranasal route was used because it provides efficient infection via the nasal associated lymphoid tissue without the potential problem of incomplete neutralization of stomach acid. Wild-type aroA\textsuperscript{+} S. enterica serovar Typhimurium strain SL2707 was used at 1 \times 10\textsuperscript{8} bacteria, lytB\textsuperscript{+} aroA\textsuperscript{+} S. enterica SL2707 pTMV19K-C7 was used at 3 \times 10\textsuperscript{8} bacteria, and lytB aroA S. enterica SL2707 pTMV19K-C22 was used at 3 \times 10\textsuperscript{9} bacteria. Prior to immunization, baseline \gamma T cell levels of each monkey were determined by flow cytometry. After immunization, blood was collected twice a week and \gamma T cells were assessed by staining with the mAbs used for monkeys (listed above) followed by flow cytometric analysis as detailed above. To determine the levels of lambda \gamma T cells in the blood, 2–10 \mu l various anti-\gamma TCR mAbs (listed above) were mixed with 100 \mu l heparinized blood, incubated for 30 min at room temperature, washed, and then reacted with PE-goat F(ab\textsuperscript{+})\textsubscript{2}, anti-mouse IgG (H+L) secondary Ab for 30 min. After washing, residual Ig binding by the secondary antisera was blocked by incubation with 5% normal mouse serum for 15 min at room temperature. The cells were then washed and reacted with FITC–anti-CD3 mAb for 30 min at room temperature. RBCs were then lysed by resuspending in 0.5 ml OptiLyse C buffer (Beckman Coulter, Fullerton, CA) for 10 min at 4°C. The cells were then washed, stained with either Hoechst 33258 or propidium iodide to identify dead cells, and then analyzed by flow cytometry. T cell–mediated immunity V\textsubscript{y}V\textsubscript{2}T cell subsets, 100 \mu l blood was reacted with the 15D anti-V\textsubscript{y}V\textsubscript{2}V\textsubscript{6}V\textsubscript{2} secondary Ab and then blocked with normal mouse serum. The cells were then reacted with FITC–anti-CD3, allophycocyanin–anti-CD2–anti-CD28 for 60 min at room temperature, washed, and then RBCs were lysed by resuspending in 0.5 ml OptiLyse C buffer (Beckman Coulter) for 10 min at 4°C. CD4 and CD8 \alpha T cells were measured by staining with FITC–anti-CD3 mAb and then with either PE–anti-CD25 (H+L) secondary Ab or PE–anti-CD8 (5ST8.5H7; Beckman Coulter) mAbs.

**Statistical analysis**

The nonparametric Mann–Whitney U test was used for all statistical comparisons because of the potential non-Gaussian distribution of the data. Statistical analyses were done using the Prism program (version 4.0c) with p values <0.01 considered statistically significant.

**Results**

Metabolically engineering llytB aroA\textsuperscript{+} S. enterica serovar Typhimurium SL2707 to overproduce HMBPP

There are two pathways for the synthesis of the IPP and DMAPP isoprenoid precursors (Fig. 1). Most bacteria and all apicomplexan parasites use the MEP pathway to synthesize isoprenoid metabolites, whereas all mammals use the mevalonate pathway. S. enterica, a Gram-negative bacterium in the order Enterobacteriales, uses the MEP pathway, and like all mammalian cells, uses the mevalonate pathway. S. enterica, a Gram-negative bacterium in the order Enterobacteriales, uses the MEP pathway. The major stimulator of V\textsubscript{y}V\textsubscript{2}V\textsubscript{6}V\textsubscript{2} T cells in microbes using the MEP pathway is HMBPP. HMBPP is produced by GcpE (also termed HMBPP synthase or IspG) and then converted to IPP and DMAPP by LysB (also termed HMBPP reductase or IspI). To derive vaccine bacteria that overproduce HMBPP, we deleted the downstream llytB gene, causing HMBPP to accumulate. To functionally complement for this lethal mutation in the MEP pathway, genes encoding enzymes for the mevalonate isoprenoid synthetic pathway from Streptomyces sp. strain CL190 were introduced into the bacteria (Fig. 1). The mevalonate pathway genes were cloned into a high-copy-number plasmid based on the pUC plasmid. Additionally, because high levels of certain isoprenoid metabolites (e.g., 3-hydroxy-3-methylglutaryl-CoA [HMG-CoA]) are toxic to bacteria (48–50), we also cloned the mevalonate pathway genes into a low-copy-number plasmid (51) to reduce the levels of the mevalonate enzymes and their metabolites. Using this approach (detailed in Supplemental Fig. 1), we generated llytB deletion mutants in attenuated aroA\textsuperscript{+} S. enterica serovar Typhimurium strain SL2707. A key step in deriving mutants was to grow the bacteria with fosmidomycin.
FIGURE 1. General schema for metabolic engineering of *Salmonella* vaccine bacteria to overproduce HMBPP by deletion of *lytB* (*ispH*) and complementation with the mevalonate pathway. There are two pathways for isoprenoid biosynthesis. Most eubacteria, apicomplexan protozoa, and plant chloroplasts use the MEP pathway. Most eukaryotic species, including humans and other mammals, use the mevalonate pathway. In eubacteria and parasites using the MEP pathway, *V*.-*m*ethyl-*D*-erythritol-4-phosphate reductase (52) in the MEP pathway. Blocking the MEP pathway switches bacterial isoprenoid synthesis to the introduced *HMGB reductase*.

After transforming the bacteria with plasmids containing the mevalonate pathway allows the bacteria to survive. CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; CMS, 4-diphosphocytidyl-2-C-methylerythritol synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; HDR, HMBPP reductase; HDS, HMBPP synthase; IDI, isopentenyl diphosphate isomerase; MCS, 4-diphosphocytidyl-2-C-methyl-D-erythritol-4-phosphate synthase; MK, mevalonate kinase; MDPD, mevalonate diphosphate decarboxylase; PMK, phosphomevalonate kinase.

**Attenuated *Salmonella* enterica**

*Salmonella* SL7207 complemented with the pMMV19K low-copy-number plasmid but not with the pTMV19K high-copy-number plasmid

The introduction of the mevalonate pathway into *E. coli* can inhibit its growth owing to the overproduction of HMG-CoA (49, 50). This metabolite is not normally present in *E. coli* and inhibits fatty acid biosynthesis, leading to generalized membrane stress (49). To assess the effect of *lytB* deletion and introduction of the mevalonate pathway on the *Salmonella* vaccine bacteria, their growth was compared with the wild-type parent. Although all mutant bacteria grew at similar rates to the wild-type parent bacteria during the logarithmic growth phase, the *lytB* deletion mutants complemented with the pTMV19K high-copy-number plasmid entered stationary phase at a lower density than did the wild-type parent (∼1.3–1.4 OD₆⁰₀ for mutants versus ∼1.6 for the parent) (Fig. 3A, left panel). In contrast, one of the *lytB* deletion mutants (pMMV19K-C22) complemented with the pMMV19K low-copy-number plasmid showed identical growth as did the wild-type parent whereas two other pMMV19K clones grew to slightly lower densities (Fig. 3A, right panel). The differences in the growth of the pTMV19K clones were reflected in the morphology of the bacteria. *LytB* ara⁻ *Salmonella* SL7207 mutants complemented with the pTMV19K high-copy-number plasmid exhibited an elongated morphology with some bacteria >5 μm in length (Fig. 3B, middle panels) as compared with the short rod shape of the wild-type parent, which were all <2.5 μm (Fig. 3B, left panel). *LytB* ara⁻ *S. enterica* mutants complemented with the mevalonate pathway on the pMMV19K low-copy-number plasmid exhibited a similar morphology to the wild-type parent and were all <2.5 μm in length (Fig. 3B, right panel). Therefore, complementation of *lytB* deletion with the pTMV19K high-copy-number plasmid resulted in early entry in stationary phase and abnormal morphology whereas complementation with the pMMV19K low-copy-number plasmid resulted in mutants with similar growth and morphology to the parent strain.

**Normal invasiveness and persistence in human HeLa cells of lytB ara⁻** *S. enterica* serovar Typhimurium strain SL7207 complemented with the pMMV19K low-copy-number plasmid but not with the pTMV19K high-copy-number plasmid

The ability of *Salmonella* bacteria to invade and persist or proliferate in human cells is a major determinant of their infectivity and virulence. To assess the effect of deleting *lytB* on this ability, we tested *Salmonella* mutants for their invasiveness and persistence in human epithelial HeLa tumor cells using the standard gentamicin resistance assay (46). Invasion by *lytB* ara⁻ *S. enterica* serovar Typhimurium bacteria complemented with the high-copy-number
corresponds to an HMBPP concentration of 31.6 pM or 31.6 fmol/ml. wt, wild-type. Note that the pTMV19K mutants produced an average of 4.0

levels are maximal during early stationary phase and decrease thereafter. Again, most of the bioactivity is in the supernatant.

FIGURE 2. LytB gene deletion greatly increased the bioactivity of aroA− S. enterica serovar Typhimurium SL7207 bacteria for Vγ2Vδ2 T cells. (A) Bioactivity of Salmonella bacteria with lytB gene deletions complemented with the mevalonate pathway cloned into a high-copy-number plasmid (representative pTMV19K clones, left panel), average bioactivity of 17 pTMV19K clones as compared with the level of bioactivity of the parent SL7207 bacteria (mean of seven lots) (middle panel), or into a low-copy-number plasmid (pMMV19K clones, right panel). Bacteria were grown to early stationary phase (16–24 h), harvested, washed, and then sonicated. Bioactivity for Vγ2Vδ2 T cells was determined for culture supernatants (open portions of bars) and bacterial sonicates (filled portions of bars) using the 12G12 Vγ2Vδ2 T cell clone in an in vitro proliferation assay. One unit of bioactivity per milliliter corresponds to an HMBPP concentration of 31.6 pm or 31.6 fmol/ml. wt, wild-type. Note that the pTMV19K mutants produced an average of 4.0 × 10^6 ± 2.3 × 10^7 U/l (mean ± SD, n = 19) of bioactivity with a significant proportion of the bioactivity secreted (86 ± 14%) whereas the parent SL7207 stain produced an average of 7.171 ± 10.686 U/l (n = 7 different lots) with none of the bioactivity secreted. (B) Variation in bioactivity levels during the growth of E. coli. Note that bioactivity levels are maximal during the late stationary phase of growth when most bioactivity is in the culture supernatant. Reprinted with permission from Puan et al. (1). (C) Variation in bioactivity levels during the growth of S. enterica SL7207 pMMV19K-C22. Note that phosphoantigen levels are maximal during early stationary phase and decrease thereafter. Again, most of the bioactivity is in the supernatant.

plasmid was reduced 96% compared with wild-type aroA− S. enterica bacteria (Fig. 4, left upper panel). In contrast, invasion by the lytB− aroA− Salmonella bacteria complemented with the low-copy-number plasmid (pMMV19K-C1 and pMMV19K-C10) was identical to the wild-type parent (Fig. 4, right upper panel). Although lytB− aroA− Salmonella pTMV19K bacteria complemented with the high-copy-number plasmid invaded poorly, they were able to persist within HeLa cells (reduced 75% compared with wild-type bacteria) (Fig. 4, left lower panel), similar to lytB− aroA− Salmonella complemented with the low-copy-number plasmid or the wild-type parent (Fig. 4, right lower panel). Consistent with an earlier report (46), there was minimal proliferation of S. serovar Typhimurium bacteria in HeLa cells. Thus, by using a low-copy-number plasmid, we were able to retain normal invasiveness and persistence of lytB− aroA− S. enterica serovar Typhimurium bacteria in human cells.

Ex vivo expansion of Vγ2Vδ2 T cells from human donors upon exposure to live lytB−aroA− Salmonella bacteria

To test the ability of the Salmonella mutants to stimulate human Vγ2Vδ2 T cells in vivo, we exposed human PBMCs to live vaccine bacteria and measured the ex vivo expansion of Vγ2Vδ2 T cells. Direct infection of human PBMCs by the lytB− aroA− Salmonella bacteria pMMV19k-C22 (complemented with the low-copy-number plasmid) stimulated expansion of Vγ2Vδ2 T cells (Fig. 5, similar to that observed with direct stimulation with HMBPP or zoledronate. Because many bacteria, including Salmonella, secrete HMBPP (Fig. 1 and Refs. 1, 53, and 54), we tested the ability of the lytB− aroA− Salmonella bacteria to stimulate Vγ2Vδ2 T cells without direct contact by culturing the bacteria in the upper well of a Transwell where they were separated from the PBMCs by a 0.4-μm membrane. Despite this barrier, the lytB− aroA− Salmonella bacteria stimulated Vγ2Vδ2 T cell expansion albeit to a lesser degree than those in direct contact (Fig. 6). Thus, the vaccine bacteria were able to efficiently stimulate Vγ2Vδ2 T cell expansion ex vivo, suggesting that they would be able to stimulate Vγ2Vδ2 T cells upon in vivo immunization.

Immunization of rhesus monkeys with lytB− aroA− Salmonella bacteria complemented with the pMMV19K low-copy-number plasmid expands Vγ2Vδ2 T cells while maintaining early memory subsets

To test the effectiveness of the lytB− aroA− Salmonella bacteria in vivo, we used rhesus monkeys as an animal model for human immunization. γδ T cells that can be stimulated by prenyl pyro-
phosphates are not present in mice, rats, and other small animals, precluding their use. In contrast, rhesus monkeys and other non-human primates have reactivity to HMBPP, alkylamines, and aminobisphosphonates identical to humans (55). This reactivity is consistent with the high sequence conservation of their \( \gamma \nu \nu \) TCRs with human \( \gamma \nu \nu \) TCRs (55, 56). To assess the effectiveness of immunization, two parameters were followed: the percentage of \( \gamma \nu \nu \) T cells among total CD3 T cells before and after immunization, and the proportions of the different memory \( \gamma \nu \nu \) T cell subsets.

Memory \( \gamma \nu \nu \) T cells can be divided into early central memory, CD27+ early central memory, intermediate effector memory, and CD45RA+ late effector memory subsets based on their expression of CD28 and CD27 (C. Jin and C.T. Morita, unpublished observations and Ref. 57) similar to CD8 \( \alpha \beta \) T cells (58). Each subset has distinctive functional and migratory properties with the proportions of the subsets varying between individuals (C. Jin and C.T. Morita, unpublished observations and Ref. 57). Adoptive transfer of early central memory CD8 \( \alpha \beta \) T cells has shown that these cells are the most effective at establishing T cell memory on transfer (59) and are more effective than late effector memory T cells at mediating tumor immunity (60, 61). This is presumably due to the ability of early central memory T cells to proliferate, allowing their amplification and persistence. For this reason, we followed the proportion of early central memory \( \gamma \nu \nu \) T cells to proliferate, allowing their amplification and persistence.

This is presumably due to the ability of early central memory T cells to mediate tumor immunity (60, 61). For this reason, we followed the proportion of early central memory \( \gamma \nu \nu \) T cells to proliferate, allowing their amplification and persistence.

Figure 3. LytB gene deletion in \( aroA^- \) S. enterica serovar Typhimurium SL7207 decreased growth and altered morphology when the bacteria were complemented with the mevalonate pathway cloned into a high-copy-number plasmid but not when cloned into a low-copy-number plasmid. (A) Growth was measured for lytB deletion mutants of S. enterica serovar Typhimurium SL7207 bacteria complemented with either the high-copy-number pTMV19K plasmid (left panel) or the low-copy-number pMMV19K plasmid (right panel) as compared with the wild-type parent. (B) Abnormal morphology of Salmonella SL7207 bacterial clones with lytB gene deletion was observed when the bacteria were complemented with a high-copy-number plasmid (middle panel) compared with those complemented with a low-copy-number plasmid (right panel) or the wild-type parent (left panel). The bacteria were grown overnight on LB agar plates and then Gram-stained. Scale bar, 2.5 \( \mu \)m.
γδ T cell levels also increased significantly and these increases were more than the sum of the V61 and V62 T cells, suggesting that γδ T cells expressing alternative V genes to V61 and V62 were present. The broad nature of the response suggests that production of T cell growth cytokines (IL-2 and IL-15) might have contributed to the response. Unlike immunization with bromohydrin pyrophosphate (BrHPP, Phosphostim) (64, 65) or zoledronate (Ref. 28 and data not shown), anergy was not observed after immunization with Salmonella pMMV19K-C22 bacteria as evidenced by the expansion of Vγ2Vδ2 T cells in response to HMBPP on in vitro stimulation of PBMCs from the three monkeys (Supplemental Fig. 2). In summary, complementation of lytB deletion with mevalonate pathway genes cloned into a low-copy-number plasmid preserved invasiveness and allowed for the in vivo stimulation of Vγ2Vδ2 T cells, albeit weakly. Metabolic engineering of attenuated aroA– Salmonella bacteria to overproduce HMBPP restored their ability to stimulate expansion of Vγ2Vδ2 T cells.

**Discussion**

Development of vaccines targeting Vγ2Vδ2 and other unconventional T cells has been hampered by the rapid development of T cell anergy and T cell deletion when the stimulators are directly administered. In this study, we have used metabolic engineering to derive a live Salmonella vaccine for Vγ2Vδ2 T cells. Depletion of the lytB gene resulted in the accumulation of its substrate, HMBPP, to high levels compared with the low levels found in the parent vaccine strain. Because this mutation is lethal, the loss of lytB was complemented with a plasmid expressing mevalonate pathway enzymes from Streptomyces. It was crucial to control the levels of the enzymes to minimize the production of toxic intermediates such as HMG-CoA that caused early entry into stationary phase and that inhibited bacterial invasiveness for human cells. LytB–aroA– Salmonella complemented with the mevalonate pathway on a low-copy-number plasmid expanded human Vγ2Vδ2 T cells cross-reactive with Salmonella serovar Typhimurium vaccine bacteria pMMV19K-C22 at an MOI of 1:1. After 6 h, the cells were washed and then cultured in the presence of IL-2 with antibiotics added to kill extracellular bacteria. After 9 d, the cells were harvested and analyzed by flow cytometry. Data are representative of at least two experiments for each donor except for donor 7 where one experiment was done. The bars show the percentage of CD3 T cells expressing different V genes or γδ TCRs at the end of the culture with or without the bacteria. (B) Ex vivo expansion of Vγ2Vδ2 T cells upon exposure to live lytB–aroA– Salmonella pMMV19K-C22 bacteria (24.7 ± 12.8%) compared with media control (2.2 ± 1.1%). Data shown are the results of seven normal human donors with the means and SDs shown as lines. For donor 1 (■), the mean of six experiments is shown. *p = 0.0006, Mann–Whitney U test.

FIGURE 4. LytB gene deletion in aroA– S. enterica serovar Typhimurium SL7207 decreased invasiveness for human HeLa cells when the bacteria were complemented with the mevalonate pathway cloned into a high-copy-number plasmid but not when cloned into a low-copy-number plasmid. HeLa cells were cultured with the various bacterial mutants for 2 h, washed, and either lysed after culturing for 2 h with high-dose gentamicin (100 µg/ml) (to measure invasiveness) or lysed after culturing for an additional 20 h with media containing low-dose gentamicin (25 µg/ml) (to measure intracellular survival). CFUs were then determined for the HeLa cell lysates to count surviving bacteria. Left panels show numbers of lytB–aroA– Salmonella pTMV19K-C7 mutant bacteria complemented with the high-copy-number plasmid as compared with wild-type bacteria at 2 and 24 h postinfection. Right panels show numbers of lytB–aroA– S. enterica serovar Typhimurium mutant bacteria (pMMV19K-C1 and pMMV19K-C10) complemented with mevalonate pathway genes cloned into a low-copy-number plasmid as compared with wild-type bacteria at 2 and 24 h postinfection.

FIGURE 5. Ex vivo exposure of human PBMCs to live lytB–aroA– S. enterica serovar Typhimurium vaccine bacteria pMMV19K-C22 expanded Vγ2Vδ2 T cells. (A) Ex vivo exposure of human PBMCs to live lytB–aroA– S. enterica serovar Typhimurium vaccine bacteria expands Vγ2Vδ2 T cells. PBMCs from healthy human donors were infected directly with live lytB–aroA– S. enterica serovar Typhimurium vaccine bacteria pMMV19K-C22 at an MOI of 1:1. After 6 h, the cells were washed and then cultured in the presence of IL-2 with antibiotics added to kill extracellular bacteria. After 9 d, the cells were harvested and analyzed by flow cytometry. Data are representative of at least two experiments for each donor except for donor 7 where one experiment was done. The bars show the percentage of CD3 T cells expressing different V genes or γδ TCRs at the end of the culture with or without the bacteria. (B) Ex vivo expansion of Vγ2Vδ2 T cells upon exposure to live lytB–aroA– Salmonella pMMV19K-C22 bacteria (24.7 ± 12.8%) compared with media control (2.2 ± 1.1%). Data shown are the results of seven normal human donors with the means and SDs shown as lines. For donor 1 (■), the mean of six experiments is shown. *p = 0.0006, Mann–Whitney U test.
with HMBPP/IL-2 after a prolonged rest period (35 wk) did little to restore responsiveness with expansions of 3- to 10-fold (data not shown). Thus, the loss of Vγ2Vδ2 T cell responsiveness with direct immunization with stimulating compounds is a major problem limiting the potential effectiveness of these cells in cancer immunotherapy.

Given these results, we pursued a different approach and have now developed a live bacterial vaccine that stimulates Vγ2Vδ2 T cells. We predicted that such a vaccine might be significantly better at stimulating Vγ2Vδ2 T cell immunity because it mimics the natural response of Vγ2Vδ2 T cells to infections. Vγ2Vδ2 T cells expand to large number in response to a number of different microbial infections (reviewed in Ref. 15). For example, in infections with Salmonella, γδ T cells increase from 4.5% to an average of 27.1% of total T cells in patients with typhoid/paratyphoid fever or to 12.35% for patients with serovars causing gastroenteritis (Fig. 8) (62, 63). Similar expansions are noted with infections with Listeria monocytogenes (67, 68) and with Brucella melitensis after ingestion of contaminated milk (69).

Moreover, during tularemia infection (due to Francisella tularensis), circulating Vγ2Vδ2 T cells increase as early as 1 wk with levels 2 wk postinfection between 22 and 50% of the circulating T cells (70). These increases persist for >1 y (70). Similar expansions to bacterial infections are also noted in nonhuman primates. In collaborative studies performed by the Z.W. Chen laboratory, we found strong evidence that Vγ2Vδ2 T cells mount adaptive immune responses to mycobacterial infections. Whereas the initial infection of rhesus monkeys with Mycobacterium bovis bacillus Calmette-Guérin (BCG) leads to Vγ2Vδ2 T cell expansions that peak on day 29, reinduction leads to earlier responses that peak on day 19 with higher numbers of Vγ2Vδ2 T cells (29). Similar recall responses are also observed with i.v. infection with attenuated L. monocytogenes (30). These results suggest that bacterial infections lead to the development of Vγ2Vδ2 T cell memory responses. The development of CD4 γδ T cell immu-
nity to provide T cell help, the conversion of the remaining naive Vγ2Vδ2 T cells to memory Vγ2Vδ2 T cells, and the activation of the innate immune system probably account for the boost in the Vγ2Vδ2 T cell response.

In contrast to pathogenic bacterial infections, vaccination of humans with live attenuated bacterial vaccines in clinical use has not been found to expand Vγ2Vδ2 T cells. No expansion of circulating Vγ2Vδ2 T cells was found on immunization with F. tularensis LVS to prevent tularemia (70) and only slight increases in some monkeys on immunization with BCG to prevent tuberculosis (71), whereas virulent F. tularensis (70) and Mycobacterium tuberculosis (16, 29, 72) do expand Vγ2Vδ2 T cells on infection. Vγ2Vδ2 T cell expansions are also observed in rhesus monkeys in response to i.v. infection with attenuated L. monocytogenes ΔactA prfA, but this vaccine is not licensed for human use and the i.v. route would not be amenable for mass immunizations (30). The effectiveness of Listeria may reflect the fact that attenuation in this strain is due to alterations in virulence rather than in metabolism and that the bacteria were given i.v., causing bacteremia. Although intradermal BCG immunization did not greatly expand circulating Vγ2Vδ2 T cells, it partially stimulates Vγ2Vδ2 T cell immunity as evidenced by increases in the in vivo responsiveness of blood Vγ2Vδ2 T cells to mycobacteria (71, 73, 74).

The inability of the licensed attenuated bacterial vaccines to expand Vγ2Vδ2 T cells likely reflects the fact that both F. tularensis LVS (70) and BCG (75) produce lower levels of stimulatory compounds (e.g., HMBPP) than do the comparable pathogenic bacteria. Similarly, we found that bioactivity for Vγ2Vδ2 T cells was very low in the attenuated aroA S. enterica serovar Typhimurium
SL7207 vaccine strain used in the present study (Fig. 2). Because we and others have found that the deletion of the lytB gene in E. coli greatly increases HMBPP levels (1, 2, 76), we decided to delete the lytB gene from Salmonella in an attempt to develop an attenuated bacterial vaccine that retained its ability to expand V\textsubscript{g}2V\textsubscript{d}2 T cells. The ability of lytB\textsuperscript{2}aroA\textsuperscript{2} Salmonella bacteria to expand V\textsubscript{g}2V\textsubscript{d}2 T cells in rhesus monkeys upon immunization demonstrates the success of this strategy.

The use of attenuated Salmonella as the basis for this new V\textsubscript{g}2V\textsubscript{d}2 T cell vaccine was guided by the success of present Salmonella vaccines. Live Salmonella vaccines have been successfully developed for use in humans as well as cattle, chickens, and other farm animals. A variety of different attenuation mutations affecting pathogenicity or metabolism have been made to develop Salmonella as a platform to allow immunization against a number of different protein Ags to vaccinate against other bacteria, viruses, and cancer Ags (77–80). In this study, we used S. enterica serovar Typhimurium with an aroA deletion mutation that is deficient in the synthesis of aromatic amino acids and folic acid (81). Salmonella serovar Typhimurium that has deletions in aromatic acid biosynthesis alone or with other deletions had prolonged fecal shedding when given to some volunteers (82) (although not in rhesus monkeys) (83), whereas serovar Typhi caused silent bacteremia but only short periods of fecal shedding (84). Thus, for the development of a clinical V\textsubscript{g}2V\textsubscript{d}2 T cell vaccine, the lytB mutation might need to be made in a more attenuated Salmonella strain than SL7207.

An additional advantage of using a metabolically engineered Salmonella bacteria with a lytB deletion is that variants that lose production of HMBPP are unlikely to develop. The MEP pathway is essential in Salmonella as evidenced by the fact that lethal point mutations were found in all of the MEP pathway enzymes, including lytB (85), when S. enterica bacteria that had been transformed with a synthetic operon containing downstream yeast mevalonate pathway enzymes were chemically mutagenized. Additionally, we and others showed that deletion of any of the MEP pathway enzymes,
FIGURE 8. Response of Vγ2Vδ2 T cells to Salmonella infection differs between vaccines/serovars causing localized mucosal infections and serovars causing systemic infections. The maximum γδ T cell levels observed after primary and secondary immunization of theseus monkeys with the serovar Typhimurium pMMV19K-C22 vaccine are compared with γδ T cell levels observed after human infections with different serovars. The data for human infections are from Hara et al. (62) and Hoshina et al. (63) with additional patient information provided by T. Hara (personal communication). The maximum γδ T cell level for each patient is shown. Serotyping was based on identification of bacteria cultured from stool or blood samples. Note that in two Paratyphi B cases, the bacteria were only isolated from stool samples but are included in the Typh/Paratyphi group. The mean levels are shown as dashes.

Isoprenoid compounds (also termed terpenoids) are among the largest and most diverse group of natural organic compounds. Despite their variety and complexity, all of these compounds are derived from IPP and DMAPP precursors. Given the importance of many of these compounds as drugs, plant products, and fuels, efforts have been made to alter isoprenoid metabolism in bacteria by the introduction of new enzymes or other modifications to increase the overall metabolic flux through the isoprenoid pathway (33, 48, 92) and to allow for the synthesis of complex isoprenoid compounds (48, 93). However, to our knowledge, this is the first report using metabolic engineering to develop a vaccine for unconventional T cells.

Alteration of isoprenoid metabolism by the introduction of mevalonate pathway enzymes can lead to the overproduction of toxic metabolic intermediates. Isoprenoid metabolism is normally tightly regulated to avoid this, but regulation can be lost with the introduction of heterologous enzymes as was noted in this study. When the mevalonate pathway was introduced into Salmonella using the pMMV19K high-copy-number plasmid, the bacteria entered stationary phase early and exhibited an abnormal elongated morphology. It also led to their inability to efficiently invade human cells and to expand monkey Vγ2Vδ2 T cells upon in vivo immunization. These defects are likely due to the overproduction of HMG-CoA given that the accumulation of this metabolite was found to correlate with decreased growth in E. coli engineered by the introduction of yeast mevalonate pathway genes (50). Overproduction of HMG-CoA inhibits fatty acid biosynthesis, leading to generalized membrane stress and upregulation of bacterial stress response genes (49). We were able to overcome toxicity by using the low-copy-number pMW118 plasmid that is limited to fewer than five copies per bacteria. The similar use of low-copy-number plasmids allowed cloning of a mevalonate gene cluster from Streptomyces griseolosporus (94) and improved isoprenoid product yields (51). Thus, regulation of isoprenoid biosynthesis is critical to restore sufficient virulence to the lytB araA−Salmonella for effective immunization.

What are the potential uses of a live S. enterica vaccine that stimulates Vγ2Vδ2 T cells? One use would be to stimulate Vγ2Vδ2 T cells for immunotherapy of tumors. A live Salmonella vaccine could be given to immunocompetent patients with cancer or as adjuvant therapy for patients after treatment or be used to expand Vγ2Vδ2 T cells ex vivo for use in adoptive immunotherapy. Finally, Salmonella preferentially accumulates in tumors in experiments in mice and is tumoricidal (95, 96), so therapy with attenuated Salmonella with deletion of lipid A to avoid septic shock (97) has been proposed. Although insufficient tumor colonization was noted in a clinical trial (98), recent vaccines have engineered Salmonella to express antitumor Abs to target them directly to tumors (99). Similar treatment of a lytB mutant Salmonella to localize it to tumors could be used to attract adoptively transferred Vγ2Vδ2 T cells to tumors and activate them.

A second use would be to prime Vγ2Vδ2 T cell immunity in infants and adults to increase protection from bacterial and protozoan infections. Vγ2Vδ2 T cells play an important role in primary immunity to bacteria and apicomplexan parasites. Once primed, Vγ2Vδ2 T cells have the unique ability to mount memory responses to bacteria and protozoa unrelated to previous infections except for the production of the HMBPP metabolite in the MEP isoprenoid pathway. Vγ2Vδ2 T cells likely play an important role in containing infections in their early phases, thereby gaining time for the establishment of microbe-specific γδ T cell and B cell responses to provide sterilizing immunity. For example, in tuberculosis in nonhuman primates, stimulation of Vγ2Vδ2 T cells by an HMBPP analog decreased M. tuberculosis numbers in the lung by ~75–90% with less severe lung damage (100). In vitro experiments, Vγ2Vδ2 T cells decrease intracellular numbers of M. tuberculosis (100, 101), M. bovis BCG (102, 103), L. monocytogenes (30), and Brucella suis (104, 105). Control of bacterial numbers can be through several mechanisms, including perforin (100), granzyme A (103), or Fas/Fas ligand (106) mediated lysis of the infected cell followed by direct killing of the bacteria by...
efficient prenyl pyrophosphate stimulation of V

1. inheritance of the relative proportions of
tical twins can differ and there was no evidence in families for in-
inherited but is driven by environmental factors (111). Thus, iden-

2. influence of environmental factors because expansion of cord blood
to the enrichment for adult-like V

3. mental factors shape the normal
gd
t cells is almost entirely due to the expansion of V
4. adult T cells predominate by a two to one margin. Moreover, the ¥5 T cell
cell population only constitutes 1.7% of total T cells. In contrast, in
t adults the V
5. T cell population constitutes 51% of ¥5 T cells, and ¥5 T cells constitute 4.8% of total T cells (110). This increase in
6. T cells is almost entirely due to the expansion of V
7. T cells to ~4.5% of total T cells between ages 1 and 10 y, leading to their

8. progeny among ¥5 T cells in adults (111). This expansion of
9. T cells is not present in every individual and is not

10. identical twins can differ and there was no evidence in families for in-
heritance of the relative proportions of ¥5 T cell gene subsets (111). Sequence analysis of V
11. 2 TCRs (112) provides support for the influence of environmental factors because expansion of cord blood
12. T cells by stimulation with an aminobisphosphonate leads to the enrichment for adult-like ¥2 T cells, selecting support for

13. efficient prenyl pyrophosphate stimulation of V
14. T cells (113). Studies of rhesus monkeys provide further evidence that environ-
mental factors shape the normal ¥5 T cell V gene repertoire. Unlike
15. human adults but similar to human infants, adolescent and adult
16. rhesus monkeys raised in captivity in specific pathogen-free colonies do not have V
17. T cells as the predominant ¥5 T cell subset. Instead, ¥5 T cells are the major subset constituting 32% of total
18. ¥5 T cells, whereas V
19. T cells constitute only 24% (55). Thus, without proper environmental cues, normal development of V
20. T cell immunity does not occur. A live Salmonella vaccine could be used to prime V
21. T cell immunity in young infants rather than waiting for a natural infection that might cause illness or death. This
22. might be especially useful in underdeveloped nations where bacterial
gastroenteritis, tuberculosis, and malaria (caused by HMBPP-
producing apicomplexan protozoa) are major causes of infant mor-
bidity and mortality.

23. Although increases in V
24. T cell levels were noted after immunization with Salmonella pMMV19K-C22 vaccine bacteria, these increases were relatively modest compared with the high
25. levels that occur with certain infections (reviewed in Ref. 15). However, the V
26. T cell levels achieved by one monkey (6.3% in monkey RQ5234) reached the mean levels noted in human infants when V
27. T cells are expanding (111), suggest-
ging that the vaccine response may be sufficient to prime V
28. T cell immunity. One potential drawback was the vari-
ability in the response with one monkey not responding. This may

29. reflect natural variability in the response given that even infections with virulent serovar Typhimurium bacteria do not uniformly

30. induce V
31. T cell responses (Fig. 8). Moreover, the levels found in the monkeys were similar to those reported for
32. patients with infections with other pathogenic S. enterica serovars that cause gastroenteritis (Fig. 8) (62, 63). Of 28 patients with
gastroenteritis, 13 (46%) had ¥5 T cells <10% of total T cells with mean values of 12.4%. In contrast, systemic infection with
33. serovars Typhi and Paratyphi had uniformly higher levels of ¥5 T cells (mean of 27.1%) (Fig. 8). Because serovar Typhi bacteria

34. only infect great apes (Hominidae family), we chose to use an attenuated Typhimurium strain to allow direct testing in lower
35. primates and virulence testing in mice. However, based on the results presented in the present study and the differences in V
36. T cells in patients infected with different Salmonella serovars, it is likely that a lysB deletion mutant in an attenuated Typhi serovar strain

37. would induce higher V
38. T cell expansions. This will be the focus of our future vaccine efforts because the responses induced by the
39. pMMV19K-C22 serovar Typhimurium vaccine are likely to be too variable to be of significant benefit. Our findings further suggest that
40. systemic bacterial and protozoan parasite infections are the most effective at expanding V
41. T cells compared with local infec-
tions in the mucosa or skin. This is consistent with the presence of
42. resting V
43. T cells primarily in central lymphoid organs and in the
44. blood rather than being concentrated in the intestinal mucosa or

45. skin (114). Thus, initial activation of V
46. T cells likely occurs in the spleen and lymph nodes. After expansion, V
47. T cells can then migrate in high numbers to peripheral sites to provide local immunity (16, 29, 30).

48. In conclusion, genetic engineering of vaccine bacteria has fo-
cused on the deletion of virulence genes, on the deletion of genes

49. important in bacterial metabolism to decrease virulence and/or

50. survival, or on the introduction of heterologous proteins or protein

51. fragments to induce immune responses to other microbes or to
cancer cells. However, we now show that genetic engineering can

52. also be used to alter the metabolism of vaccine bacteria to increase

53. production of nonpeptide compounds that are stimulators/Ags for unconventional T cells. A similar approach could be used to de-

54. velop vaccines for other unconventional T cells. ¥5 iNKT cells

55. recognize specific bacterial lipids presented by CD1d that are produced by Sphingomonas spp. (115) and Borrelia burgdorferi

56. (116). The enzymes required to produce a stimulatory glycolipid

57. could be introduced into attenuated Salmonella to allow them to

58. stimulate iNKT cells. Metabolic engineering of B vitamin syn-
thetic pathways could similarly be performed to increase the production of the metabolites (117, 118) that are recognized by

59. ¥5 mucosal-associated invariant T cells (119, 120), thereby

60. increasing the ability of Salmonella vaccine bacteria to stimulate mucosal-associated invariant T cell immunity. Altering the poly-

61. saccharides of vaccine bacteria could also be used to stimulate Ab

62. formation against unique microbial carbohydrate structures (121). Finally, introduction of pathways involved in the synthesis of TLR

63. ligands or ligands for other innate receptors could be used to in-
crease the immunogenicity of the bacteria without increasing their

64. virulence. Altering the metabolism of bacteria to produce non-

65. peptide stimulators/Ags for T cells or nonpeptide ligands for

66. innate receptors could be a powerful approach for developing

67. vaccines to stimulate innate T lymphocyte immunity without in-
ducing anergy.

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C.T.M. and B.D.J. are co-inventors of U.S. patent 8,012,466 on the develop-
ment of live bacterial vaccines for activating ¥5 T cell immunity. The other
authors have no financial conflicts of interest.

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Preferential recognition of a microbial metabolite by human V
2 T cells.

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SUPPLEMENTAL FIGURE 1. Detailed schema for metabolic engineering of *Salmonella* vaccine bacteria to overproduce HMBPP by deletion of *lytB* (*ispH*) and complementation with the mevalonate pathway. The locus for the genes for mevalonate pathway enzymes was
isolated from *Streptomyces* sp. strain CL190 and cloned into either the high copy number plasmid pTTQ18 or the low copy number plasmid pMW118. The resulting plasmids (pTMV19km\(^{r}\) and pMMV19km\(^{r}\), respectively) were transformed into *aroA* \(^{-}\) *Salmonella enterica* serovar Typhimurium SL7207 vaccine bacteria to complement for the loss of the *lytB* gene. The bacteria were then transformed with a plasmid containing genes for \(\lambda\) Red recombination proteins in a temperature-sensitive plasmid (pKD46amp\(^{r}\)). The transformants were cultured at 30°C with fosmidomycin (to block the MEP pathway and switch isoprenoid metabolism to the mevalonate pathway), ampicillin (for retention of the \(\lambda\) Red plasmid), and kanamycin (as a second selection for retention of the mevalonate pathway plasmid). To target the *lytB* gene, PCR amplification with primers flanking the *lytB* gene were used to generate a 1.1 kb linear fragment encoding the chloramphenicol resistance gene flanked by 50 base pairs of 5' and 3' sequence adjacent to the *lytB* gene. L-arabinose was then added to induce the recombination enzymes and the DNA fragment targeting the *lytB* gene introduced into the bacteria by electroporation. Chloramphenicol was used to select for *lytB* deletion mutants that were then grown at 37°C to eliminate the pKD46 plasmid.
SUPPLEMENTAL FIGURE 2. HMBPP reactivity by Vγ2Vδ2 T cells is preserved after immunization with *Salmonella* pMMV19K-C22. PBMC were isolated from the blood of the RQ5234, RQ7125, and RQ5264 rhesus monkeys at various times before primary immunization and after secondary immunization with the *Salmonella* pMMV19K-C22 vaccine bacteria. PBMC were cultured with HMBPP for 10 days followed by analysis by flow cytometry. Note that the in vitro responses to HMBPP after secondary immunization are identical to those observed prior to primary immunization.