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

_J Immunol_ 2014; 193:708-721; Prepublished online 18 June 2014;
doi: 10.4049/jimmunol.1302746
http://www.jimmunol.org/content/193/2/708

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/06/18/jimmunol.1302746.DCSupplemental

**References**
This article cites 119 articles, 56 of which you can access for free at:
http://www.jimmunol.org/content/193/2/708.full#ref-list-1

**Subscription**
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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<sup>−</sup> 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 the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding the gene for LytB (the downstream enzyme from HMBPP) and functionally complementing for this loss with genes encoding

U

nconventional, 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-

Abbreviations used in this article: BCG, bacillus Calmette–Guerin; BrHPP, bromohydrin pyrophosphate; DMAPP, dimethylallyl pyrophosphate (diphosphate); HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (diphosphate); HMP, 3-hydroxy-3-methylglutaryl-CoA; INKT, invariant NKT; IPP, isopentenyl pyrophosphate (diphosphate); km, kanomycin resistance gene; LB, Luria–Bertani; MEP, 2-C-methyl-d-erythritol-4-phosphate; MOI, multiplicity of infection; OD<sub>500</sub>, OD at 600 nm.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302746

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 preyl pyrophosphates or aminobisphosphonates. The bacteria would provide the necessary adjuvants to activate innate immunity and Ags to stimulate CD4 eff 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 S. enterica enteric 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 non-typhoidal 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 avo− S. enterica serovar Typhimurium SL7207 (also termed 2337-65) 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 pSCL101 plasmid (43).

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

The lytB gene was deleted from avo− S. enterica serovar Typhimurium SL7207 by homologous recombination using the “one-step inactivation” technique with the λ Red system (44). As detailed in Supplemental Fig. 1, avo− 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 pTMV19km plasmid or the pMMV19km 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 (OD600nm) and grown with fosmidomycin and 1-aminocyclopropane-1-carboxylic acid (10 mM) (to induce the mevalonate enzymes) until reaching an OD600nm 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′-CAGTTCTAGATTTGCAATTGGAATGTTGTGAAATCGATCCGGCGCTGGAGGC-3′ and the lytB reverse primer was 5′-TTAAGCCTCTGCAATGACCCGCTGCTTCGATCTCTCTCCTTCCTTGGT-3′. The resulting 1.1-kB DNA fragment was introduced by electroporation into avo− Salmonella containing pTMV19km or pMMV19km 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 quantify 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, Edison, NJ) oscillating at 225 revolutions/min. The cultures were harvested and washed twice with Milli-Q H2O. The bacteria were then suspended in 10 ml Milli-Q H2O. The sonicates were added to the cultures at half-log dilutions and incubated at 37°C. The cultures were pulsed with 1 μCi [3H]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 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 mM.

Growth and morphology of Salmonella vaccine bacteria

Wild-type avo− S. enterica serovar Typhimurium SL7207 bacteria and lytB deletion mutants were cultured overnight and then diluted to 0.05
SALMONELLA VACCINE TO BOOST V2V82 T CELL IMMUNITY

OD_{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 37ºC. Then a colony was resuspended in H₂O₂ spread on a slide, and Gram-stained (BD Biosciences, Franklin Lakes, NJ).

Invasiveness and intracellular survival of Salmonella bacteria in human 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 10:1 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 µg/ml) for an additional 2 h, washed again, and further incubated for 20 h with media supplemented with low-dose gentamicin (25 µg/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 assesses bacterial invasiveness whereas the 24-h incubation period assesses bacterial intracellular survival.

Ex vivo expansion of human V2V82 T cells stimulated by live Salmonella bacteria

For ex vivo expansion of V2V82 T cells stimulated by live Salmonella bacteria, PBMCs were isolated from the blood or leukopacs of normal human donors by Ficoll-Hypaque density centrifugation. PBMCs (1 × 10⁶) 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 µg/ml) for 2 h, followed by low-dose gentamicin (25 µg/ml) for the remainder of the culture period. The cells were then incubated in complete RPMI 1640 media at 37ºC with 5% CO₂. On day 3, 2 nM (100 nM/ml IL-2 was added and the cells were harvested on day 9. For the Transwell assay, 1–3 × 10⁵ bacteria were added in 0.1 ml RPMI 1640 medium to the inner wells of a Transwell plate (Corning Costar, Kennebunk, ME). 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 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 36 V2V82 T cells enumerated by flow cytometric analysis using an LSRII flow cytometer and CellQuest or FACS Diva software (BD Biosciences, San Jose, CA). PBMCs were stained with anti-CD₂ (anti-TCRε; 5A6.A9), anti-CD₂ (TS2.13), anti-CD₂ (15D for rhesus monkey cells and B6 for human cells), or anti-CD₂ (7A5) (all from BD Biosciences), followed by PE–goat F(ab’)_2 anti-mouse IgG (H+L) secondary Ab (BioSource International, Camarillo, CA). After washing, residual Ig binding by the secondary antiserum was blocked by incubation with 5% normal mouse serum. 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 memory V2V82 T cell subsets. 100 nM/ml IL-2 was then reacted with 15D anti-CD26 mAb followed by PE–goat F(ab’)_2 anti-mouse IgG (H+L) secondary Ab and then blocked with normal mouse serum. The cells were then reacted with FITC–anti-CD3, allophycocyanin–anti-CD27, and PE–Cy7–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, 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 memory V2V82 T cell subsets. All animals were maintained according to 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 µl 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 S. enterica serovar Typhimurium strain SL7207 was used at 1 × 10⁷ bacteria, lytB araA S. enterica SL7207 pTMV19K-C7 was used at 3 × 10⁶ bacteria, and lytB araA S. enterica SL7207 pMMV19K-C22 was used at 3 × 10⁶ bacteria. Prior to immunization, baseline gd T cell levels of each monkey were determined by flow cytometry. After immunization, blood was drawn twice a week and gd 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 monkey gd T cells in the blood, 2–10 µl various anti-γδ TCR mAbs (listed above) were mixed with 100 µl heparinized blood, incubated for 30 min at room temperature, washed, and then reacted with PE–goat F(ab’)_2 anti-mouse IgG (H+L) secondary Ab for 30 min. After washing, residual Ig binding by the secondary antiserum 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) for 10 min at 4°C. CD4 and CD8 gd T cells were measured by staining with FITC–anti-CD3 mAb followed by either PE–anti-CD4 (M-T477, BD Biosciences) or PE–anti-CD8B (2S8.4.10D, 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 lytB araA S. enterica serovar Typhimurium SL7207 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. The major stimulator of V2V82 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 LytB (also termed HMBPP reductase or IspI). To derive vaccine bacteria that overproduce HMBPP, we deleted the downstream lytB 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 lytB deletion mutants in attenuated aroA S. enterica serovar Typhimurium strain SL7207. A key step in deriving mutants was to grow the bacteria with fosmidomycin.
T cells of CHMBPP to accumulate whereas the addition of the mevalonate pathway causes deblocking of the CHMBPP pathway (52). PMK, phosphomevalonate kinase; MDPD, erythritol 2,4-cyclodiphosphate synthase; DXR, 1-deoxy- D-xylulose 5-phosphate reductase; DXS, 1-deoxy- D-xylulose 5-phosphate synthase; CMS, 2-C-methyl- D-erythritol synthase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; HMBPP, 1,5-mevalonate 5-phosphate reductase; HDS, 1-deoxy-D-xylulose 5-phosphate synthase; HDR, HMBPP reductase; HMBPP, 2-C-methyl-D-erythritol 4-phosphate synthase; IDI, isopentenyl diphosphate isomerase; MK, mevalonate kinase; MDPD, mevalonate diphosphate decarboxylase; PMK, phosphomevalonate kinase.

after transforming the bacteria with plasmids containing the mevalonate pathway. Fosmidomycin inhibits 1-deoxy-D-xylulose 5-phosphate reductase (52) in the ME pathway. Blocking the ME pathway switches bacterial isoprenoid synthesis to the introduced mevalonate pathway, avoiding an abrupt shift upon deletion of lytB.

Deletion of lytB greatly increases the bioactivity for V2V82 T cells of arao− S. enterica serovar Typhimurium strain SL7207 bacteria

Attenuated arao− S. enterica serovar Typhimurium strain SL7207 bacteria had low levels of bioactivity for V2V82 T cells (Fig. 2A), similar to or lower than those measured in the W3110 laboratory strain of Escherichia coli (1). Deletion of the lytB gene in Salm. greatly increased bioactivity for V2V82 T cells compared with the wild-type bacteria (Fig. 2A). Mutants with lytB deletion that were complemented with the mevalonate pathway cloned into the high-copy-number plasmid (representative pTMV19K mutants are shown out of 19 mutants; Fig. 2A, left panel) produced high levels of bioactivity compared with the wild-type parent bacteria (mean values for the wild-type bacteria [mean of seven lots] and for the 19 pTMV19K mutants are shown in the middle panel of Fig. 2A). Bacteria complemented with the low-copy-number plasmid (pMMV19K C10 and C22, Fig. 2A, right panel) also produced high levels of bioactivity. Similar to mycobacteria (53) and E. coli (1), a significant proportion of the bioactivity produced by the lytB deletion mutants was present in the supernatant (open portion of bars in Fig. 2). The levels of bioactivity in the pMMV19K C22 strain peaked in early stationary phase and decreased thereafter (Fig. 2C, right panels), whereas the bioactivity in W3110 E. coli increased throughout growth and peaked in that late stationary phase of growth (Fig. 2B, left panels). The levels of HMBPP varied between different pTMV19K colonies and between different lots of the same colony, likely reflecting differences in the bacterial growth phase, culture conditions, and perhaps epigenetic differences. This level of variation in bioactivity was also noted for E. coli (data not shown).

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 deleting lytB and deletion 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− Salm. 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 bacteria complemented with the pMMV19K low-copy-number plasmid but not with the pTMV19K high-copy-number plasmid

The ability of Salm. bacteria to invade and persist or proli ferate in human cells is a major determinant of their infectivity and virulence. To assess the effect of deleting lytB on this ability, we tested Salm. 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
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 assess 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 Vγ2Vδ2 TCRs with human Vγ2Vδ2 TCRs (55, 56). To assess the effectiveness of immunization, two parameters were followed: the percentage of Vγ2Vδ2 T cells among total CD3 T cells before and after immunization, and the proportions of the different memory Vγ2Vδ2 T cell subsets.

Memory Vγ2Vδ2 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 αβ 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 αβ 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 Vγ2Vδ2 T cells to assess the effect of immunization with Salmonella bacteria on these immune cells.

Immunization with the attenuated aroA− S. enterica serovar Typhimurium strain SL7207 parent bacteria was studied first. Unlike infection with pathogenic Salmonella (62, 63), immunization with the attenuated aroA− Salmonella bacteria did not stimulate significant expansion of Vγ2Vδ2 T cells (Fig. 7A, left panel) nor did it alter the proportions of the Vγ2Vδ2 T cell memory subsets (Fig. 7A, right panel). Because aroA− Salmonella bacteria did not stimulate Vγ2Vδ2 T cells in vivo, we next assessed the ability of lytB− aroA− Salmonella pTMV19K-C7 (complemented with a high-copy-number plasmid) to stimulate Vγ2Vδ2 T cell immunity. Although pTMV19K-C7 bacteria do not efficiently infect mammalian cells, they do produce high levels of HMBPP that could stimulate Vγ2Vδ2 T cell responses. To determine which effect predominated, a rhesus monkey was immunized with pTMV19K-C7 bacteria. Immunization did not increase Vγ2Vδ2 T cells (Fig. 7B, left panel), nor were there any changes in the proportion of Vγ2Vδ2 T cell memory subsets (Fig. 7B, right panel).

Given that the SL7207 and pTMV19K-C7 bacteria had minimal effects, lytB− aroA− Salmonella pMMV19K-C22 bacteria complemented with the low-copy-number plasmid were tested. Immunization with pMMV19K-C22 bacteria resulted in modest expansions of Vγ2Vδ2 T cells in two out of three monkeys. In monkey RQS234, Vγ2Vδ2 T cells increased to 5.6% (2.3-fold) on primary immunization and to 4.6% of total T cells on secondary immunization (Fig. 7C, left upper panels). There were no significant changes in the memory subsets that accompanied these responses (Fig. 7C, right upper panels). Elevated numbers of Vγ2Vδ2 T cells persisted until the second immunization (96 d after the first immunization). A second monkey, RQT125 (Fig. 7C, left middle panels), did not respond on primary immunization, but did respond on secondary immunization to 2.4%. Again, there were no significant changes in the Vγ2Vδ2 T cell memory subsets (Fig. 7C, left middle panels). A third monkey, RQS264, did not respond to either immunization. Although the initial Vγ2Vδ2 T cell response by monkey RQS234 did not have associated increases in the level of Vδ1 T cells, the Vγ2Vδ2 T cell responses to secondary immunization for both monkey RQS234 and RQT125 did have slight increases (Fig. 7C, left upper and middle panels). Moreover, total

![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 μm.](http://www.jimmunol.org/content/images/713/3-713-f03.png)
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.

\[\text{FIGURE 4.} \]

\textbf{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.

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 ex vivo. When tested in rhesus monkeys, immunization with the vaccine expanded Vγ2Vδ2 T cells with elevated levels of these cells persisting for at least 3 mo in one monkey. The levels of expansion were of lower magnitude compared with those noted during *Salmonella*-induced gastroenteritis in humans (10.8 ± 8.5 and 13.6 ± 9.0% in Refs. 62 and 63, respectively).

Immunization with live bacterial vaccines for Vγ2Vδ2 T cells has significant advantages over immunization with prenyl pyro-
whereas the second immunization given 2 mo later resulted in g
g" neighbors monkeys, the initial zoledronate/IL-2 immunization resul-
d to 2% by the fourth immunization (26). In our studies in g
such there were no further responses and V
gexpansion of V
gsimilarly, a patient with renal cell carcinoma exhibited an initial
V
gT cells from baseline were observed in four of the
2V
t2 T cell immunity as evidenced by increases in the in vitro
responsiveness of blood V
gT cells likely reflects the fact that both
2 T cells likely reflects the fact that both
2 T cell immunity because it mimics
the natural response of V
gT cells to infections. V
gT cells expand to large number in response to a number of dif-
ferent 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 caus-
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 tulare-
ensis), circulating V
gT 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 V
gT cells mount
adaptive immune responses to mycobacterial infections. Whereas
the initial infection of rhesus monkeys with Mycobacterium bovis
bacillus Calmette-Guérin (BCG) leads to V
gT cell expansions that peak on day 29, reinfection leads to earlier responses that peak on day 19 with higher numbers of V
gT 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 V
gT cell memory responses. The development of CD4 αβ T cell
immunity to provide T cell help, the conversion of the remaining naive
V
gT cells to memory V
gT cells, and the activation of the innate immune system probably account for the boost in the
V
gT 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 V
gT cells. No expansion of cir-
culating V
gT 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 Mycobacte-
rium tuberculosis (16, 29, 72) do expand V
gT cells on
infection. V
gT cell expansions are also observed in rhesus
monkeys in response to i.v. infection with attenuated L. mono-
cytogenes ΔactA prf4, but this vaccine is not licensed for human
use and the i.v. route would not be amenable for mass immuni-
izations (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 V
gT cells, it partially stimulates
V
gT cell immunity as evidenced by increases in the in vitro
responsiveness of blood V
gT cells to mycobacteria (71, 73, 74).

The inability of the licensed attenuated bacterial vaccines to
expand V
gT 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 bac-
teria. Similarly, we found that bioactivity for V
gT cells was very low in the attenuated aroA− S. enterica serovar Typhimurium

pMMV19K-C22 + PBMC

\[ \text{Separate} \]

\[ \text{Together} \]

\[ \text{Donor 1} \]

\[ \text{Donor 2} \]

\[ \text{Donor 3} \]

\[ \text{Donor 6} \]

\[ \% \text{ of CD3}^+ \text{T Cells} \]

\[ \text{Control mAb} \]

\[ \text{anti-γδ TCR} \]

\[ \text{anti-Vδ1 TCR} \]

\[ \text{anti-Vδ2 TCR} \]

\[ \text{anti-Vγ2 TCR} \]

\[ \text{FIGURE 6. Ex vivo expansion of human blood VγVδ T cells in re-
spose to live lytB ΔaroA− S. enterica serovar Typhimurium pMMV19K-
C22 does not require direct contact between bacteria and cells. PBMCs
from healthy human donors were either infected with lytB ΔaroA− S.
enterica serovar Typhimurium vaccine bacteria (pMMV19K-C22) in a 1:1
ratio or cultured separated from the bacteria by a 0.4-μm membrane. After
6 h, the bacteria-containing inserts were removed from cultures where the
cells were separated from bacteria or else the cells were washed and antibi-
otics added to kill extracellular bacteria for cultures where the cells
were in direct contact with bacteria. On day 3, 2 nM IL-2 was added to all
cultures and the media were changed every 3 d. After 9 d, the cells were
washed and analyzed by flow cytometry.} \]
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 Vγ2Vδ2 T cells. The ability of lytB2aroA2 Salmonella bacteria to expand Vγ2Vδ2 T cells in rhesus monkeys upon immunization demonstrates the success of this strategy.

The use of attenuated Salmonella as the basis for this new Vγ2Vδ2 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 Vγ2Vδ2 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,
The production 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 griseoluspora (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 pri-

mate 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 αβ 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 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

### 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 Typhi/Paratyphi group. The mean levels are shown as shown as dashes.
granulolysin (100, 101), through activation of infected monocytes by TNF-α (103) and IFN-γ (104), or by the production of anti-bacterial peptides such as the cathelicidin LL-37 (105) or elafin (107). Besides reducing intracellular bacterial replication and killing infected cells, Vγ2Vδ2 T cells can also traffic to infected organs and release an array of inflammatory cytokines and chemokines while also producing growth factors to speed healing of epithelial and endothelial damage (reviewed in Refs. 15, 108, 109).

At birth in the United States, we found that the Vγ2Vδ2 T cell subset is a minor subset, making up only 9.4% of γδ T cells with as many Vγ2Vδ1 and Vγ1Vδ2 T cells as Vγ2Vδ2 T cells (110). Vδ1 T cells predominated by a two to one margin. Moreover, the γδ T cell population only constitutes 1.7% of total T cells. In contrast, in adults the Vγ2Vδ2 T cell population constitutes 51% of γδ T cells, and γδ6 T cells constitute 4.8% of total T cells (110). This increase in γδ T cells is almost entirely due to the expansion of Vγ2Vδ2 T cells to ~4.5% of total T cells between ages 1 and 10 y, leading to their predominance among γδ T cells in adults (111). This expansion of Vγ2Vδ2 T cells is not present in every individual and is not inherited but is driven by environmental factors (111). Thus, identical twins can differ and there was no evidence in families for inheritance of the relative proportions of γδ V gene subsets (111). Sequence analysis of Vγ2Vδ2 TCRs (112) provides support for the influence of environmental factors because expansion of cord blood Vγ2Vδ2 T cells by stimulation with an aminobisphosphonate leads to the enrichment for adult-like Vγ2 chains, supporting selection for efficient prenyl pyrophosphate stimulation of Vγ2Vδ2 T cells (113).

Studies of rhesus monkeys provide further evidence that environmental factors shape the normal γδ T cell V gene repertoire. Unlike human adults but similar to human infants, adolescent and adult rhesus monkeys raised in captivity in specific pathogen-free colonies do not have Vγ2Vδ2 T cells as the predominant γδ T cell subset. Instead, Vδ1 T cells are the major subset constituting 32% of total γδ T cells, whereas Vγ2Vδ2 T cells constitute only 24% (55). Thus, without proper environmental cues, normal development of Vγ2Vδ2 T cell immunity does not occur. A live Salmonella vaccine could be used to prime Vγ2Vδ2 T cell immunity in young infants rather than waiting for a natural infection that might cause illness or death. This might be especially useful in underdeveloped nations where bacterial gastroenteritis, tuberculosis, and malaria (caused by HMBPP-producing apicomplexan protozoa) are major causes of infant morbidity and mortality.

Although increases in Vγ2Vδ2 T cell levels were noted after immunization with Salmonella pMMV19K-C22 vaccine bacteria, these increases were relatively modest compared with the high levels that occur with certain infections (reviewed in Ref. 15). However, the Vγ2Vδ2 T cell levels achieved by one monkey (6.3% in monkey RQ5234) reached the mean levels noted in human infants when Vγ2Vδ2 T cells are expanding (111), suggesting that the vaccine response may be sufficient to prime Vγ2Vδ2 T cell immunity. One potential drawback was the variability in the response with one monkey not responding. This may reflect natural variability in the response given that even infections with virulent serovar Typhimurium bacteria do not uniformly induce Vγ2Vδ2 T cell responses (Fig. 8). Moreover, the levels found in the monkeys were similar to the values reported for production of other pathogenic Salmonella enterica serovars that cause gastroenteritis (Fig. 8) (62, 63). Of 28 patients with gastroenteritis, 13 (46%) had γδ T cells < 10% of total T cells with mean values of 12.4%. In contrast, systemic infection with serovars Typhi and Paratyphi had uniformly higher levels of γδ T cells (mean of 27.1%) (Fig. 8). Because serovar Typhi bacteria only infect great apes (Hominidae family), we chose to use an attenuated Typhimurium strain to allow direct testing in lower primates and virulence testing in mice. However, based on the results presented in the present study and the differences in Vγ2Vδ2 T cells in patients infected with different Salmonella serovars, it is likely that a lvtB deletion mutant in an attenuated Typhi serovar strain would induce higher Vγ2Vδ2 T cell expansions. This will be the focus of our future vaccine efforts because the responses induced by the pMMV19K-C22 serovar Typhimurium vaccine are likely to be too variable to be of significant benefit. Our findings further suggest that systemic bacterial and protozoan parasite infections are the most effective at expanding Vγ2Vδ2 T cells compared with local infections in the mucosa or skin. This is consistent with the presence of resting Vγ2Vδ2 T cells primarily in central lymphoid organs and in the blood rather than being concentrated in the intestinal mucosa or skin (114). Thus, initial activation of Vγ2Vδ2 T cells likely occurs in the spleen and lymph nodes. After expansion, Vγ2Vδ2 T cells can then migrate in high numbers to peripheral sites to provide local immunity (16, 29, 30).

In conclusion, genetic engineering of vaccine bacteria has focused on the deletion of virulence genes, on the deletion of genes important in bacterial metabolism to decrease virulence and/or survival, or on the introduction of heterologous proteins or protein fragments to induce immune responses to other microbes or to cancer cells. However, we now show that genetic engineering can also be used to alter the metabolism of vaccine bacteria to increase production of nonpeptide compounds that are stimulators/Ags for unconventional T cells. A similar approach could be used to develop vaccines for other unconventional T cells. αβ iNKT cells recognize specific bacterial lipids presented by CD1d that are produced by Sphinomonas spp. (115) and Borrelia burgdorferi (116). The enzymes required to produce a stimulatory glycolipid could be introduced into attenuated Salmonella to allow them to stimulate iNKT cells. Metabolic engineering of B vitamin synthetic pathways could similarly be performed to increase the production of the metabolites (117, 118) that are recognized by αβ mucosal-associated invariant T cells (119, 120), thereby increasing the ability of Salmonella vaccine bacteria to stimulate mucosal-associated invariant T cell immunity. Altering the polysaccharides of vaccine bacteria could also be used to stimulate Ab formation against unique microbial carbohydrate structures (121). Finally, introduction of pathways involved in the synthesis of TLR ligands or ligands for other innate receptors could be used to increase the immunogenicity of the bacteria without increasing their virulence. Altering the metabolism of bacteria to produce nonpeptide stimulators/Ags for T cells or nonpeptide ligands for innate receptors could be a powerful approach for developing vaccines to stimulate innate T lymphocyte immunity without inducing anergy.

Acknowledgments
We thank Zhimei Fang and Amy Raker for technical assistance. We thank Dr. Toshirou Hara for providing additional data on γδ T cell levels in Salmonella patients. We also thank Dr. Roy Curtiss, III, for helpful discussion.

Disclosures
C.T.M. and B.D.J. are co-inventors of U.S. patent 8,012,466 on the development of live bacterial vaccines for activating γδ T cells. The other authors have no financial conflicts of interest.

References


The document contains references and citations related to various scientific studies and research topics. The text is formatted in a typical scientific journal style, with names of authors, titles of papers, and pages cited. The content appears to be focused on biological and medical research, possibly involving immunology and vaccine development. However, without the ability to read the specific content of each reference, it's difficult to extract the exact nature of the research topics covered.


