Multieffector-Functional Immune Responses of HMBPP-Specific Vγ2Vδ2 T Cells in Non-human Primates Inoculated with Listeria monocytogenes ΔactA prfA*

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Multieffector-Functional Immune Responses of HMBPP-Specific Vγ2Vδ2 T Cells in Non-human Primates Inoculated with Listeria monocytogenes ΔactA prfA*

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Although Listeria monocytogenes can induce systemic infection causing spontaneous abortion, septicemia, and meningitis, studies have not been performed to investigate human anti-L. monocytogenes immune responses, including those of Ag-specific Vγ2Vδ2 T cells, a dominant human γδ T cell subset. L. monocytogenes is the only pathogen known to possess both the mevalonate and non-mevalonate isoprenoid biosynthesis pathways that produce metabolite phosphates or phosphoantigens activating human Vγ2Vδ2 T cells, making it interesting to explore in vivo anti-L. monocytogenes immune responses of Vγ2Vδ2 T cells. In this study, we demonstrated that subclinical systemic L. monocytogenes infection of rhesus macaques via parenteral inoculation or vaccination with an attenuated Listeria strain induced multieffector-functional immune responses of phosphoantigen-specific Vγ2Vδ2 T cells. Subclinical systemic infection and reinfection with attenuated L. monocytogenes uncovered the ability of Vγ2Vδ2 T cells to mount expansion and adaptive or recall-like expansion. Expanded Vγ2Vδ2 T cells could traffic to and accumulate in the pulmonary compartment and intestinal mucosa. Expanded Vγ2Vδ2 T cells could evolve into effector cells producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after L. monocytogenes infection, and some effector Vγ2Vδ2 T cells could coproduce IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin. Surprisingly, in vivo-expanded Vγ2Vδ2 T effector cells in subclinical L. monocytogenes infection could directly lyse L. monocytogenes-infected target cells and inhibit intracellular L. monocytogenes bacteria. Thus, we present the first demonstration, to our knowledge, of multieffector-functional Vγ2Vδ2 T cell responses against L. monocytogenes. The Journal of Immunology, 2012, 189: 000–000.

Vγ2Vδ2 T cells exist only in primates, and in humans they comprise 65–90% of total circulating γδ T cells. Vγ2Vδ2 T cells can be activated by metabolites from isoprenoid synthesis, such as isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3methyl-but-2-enyl pyrophosphate (HMBPP) (1, 2), which are usually referred to as phosphoantigens. Isoprenoids are produced via two major pathways: the classical, mevalonate pathway, or the alternative, non-mevalonate pathway (2, 3). IPP is an intermediate metabolite found in both pathways, whereas HMBPP is only produced in the non-mevalonate pathway by some selected microbes including Mycobacterium tuberculosis and Listeria monocytogenes (2, 3). HMBPP is ~1000-fold more potent than IPP for in vitro activation of Vγ2Vδ2 T cells (2), and studies from us and others suggest that Vγ2Vδ2 TCR recognizes HMBPP presented by APC (4, 5). In vitro-activated Vγ2Vδ2 T cells can produce IFN-γ and TNF-α (6–9) and lyse infected cells or tumor cells via perforin (7, 10–12). In vivo, Vγ2Vδ2 T cells can mount major expansion during Mycobacterium infections, and rapid recall-like expansion of these γδ T cells after M. tuberculosis challenge of bacillus Calmette-Guérin (BCG)-vaccinated macaques is associated with BCG-induced protection against fatal tuberculosis in juvenile rhesus macaques (5). Furthermore, major expansion of Vγ2Vδ2 T effector cells after HMBPP plus IL-2 post-challenge treatment can lead to homeostatic protection against severe pneumonic plague lesions after inhalational Yersinia pestis infection of macaques (13). However, a role of Vγ2Vδ2 T cells in immunity to intracellular bacterial pathogens remains to be determined, and the definition requires in-depth studies of these HMBPP-specific Vγ2Vδ2 T cells in infections.

Listeria monocytogenes, a Gram-positive, intracellular bacterium, is the only pathogen known to possess both the mevalonate and non-mevalonate pathways of isoprenoid biosynthesis (3), making it an ideal candidate to study in vivo responses of Vγ2Vδ2 T cells and anti-L. monocytogenes effector function. Although L. monocytogenes bacteria can induce systemic infection causing spontaneous abortion, septicemia, and meningitis, anti-L. monocytogenes immunity in humans has not been defined. In murine models of systemic L. monocytogenes infection, mice deficient in neutrophils exhibit increased bacterial burden during early L. monocytogenes infection (14–16). In addition, murine IFN-γ, IL-23, IL-17, and CD8+ T cells have been shown to play a role in protection against L. monocytogenes infection (17–21). Ag-specific γδ T cell responses to L. monocytogenes infection has not been reported (22–25), and in vivo studies of Vγ2Vδ2 T cells for anti-L. monocytogenes immunity cannot be appropriately performed in mice or other laboratory animals as γδ T cells in these laboratory animals do not recognize HMBPP or other microbial Ags. Thus, non-human primate models appear to provide a unique model system in which to explore immune responses and

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Abstractions used in this article: BAA, bronchoalveolar lavage; BCG, bacillus Calmette-Guérin; DC, dendritic cell; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; ICS, intracellular cytokine staining; IPP, isopentenyl pyrophosphate; PI, propidium iodide.

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effector function of HMBPP-specific Vγ2Vδ2 T cells during L. monocytogenes infection. In the current study, rhesus macaques were infected systemically with an attenuated L. monocytogenes strain (Listeria ΔactA prfA*), which carries a deletion of the actA gene and a mutation of the constitutively active prfA gene (26, 27). The actA deletion abrogates the ability of L. monocytogenes to polymerize actin and to mediate cell-to-cell spread of L. monocytogenes, although the prfA mutation upregulates L. monocytogenes gene expression (26). The subclinical systemic Listeria ΔactA prfA* infection in macaques allowed us optimally to demonstrate multieffector-functional immune responses of Vγ2Vδ2 T cells. L. monocytogenes-activated Vγ2Vδ2 T cells could mount adaptive or recall-like expansion, traffic to or accumulate in intestinal mucosa/airways, produce multiple cytokines, and coproduce IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin. The in vivo-expanded Vγ2Vδ2 T cells can directly lyse L. monocytogenes-infected target cells and inhibit intracellular L. monocytogenes bacteria without the need for prior in vitro phospho-antigen stimulation.

Materials and Methods

Animals
Nine Chinese-origin rhesus macaques, ages 5–11, were used in this study. All animals were housed and used in accordance with the guidelines of the institutional animal care and use committee. Two macaques were infected at day 0 with 10^7 CFU i.m. attenuated Listeria ΔactA prfA* (26) and on day 35 with 10^5 CFU i.v. Seven macaques were infected at day 0 with 10^5 CFU i.v. and boosted on week 7 with 10^5 CFU i.v. Day 0 blood was drawn immediately before infection. Rectal biopsies and bronchoalveolar lavage (BAL) fluid were collected as previously described (7). Briefly, for rectal biopsies, animals were restrained in ventral recumbency with the pelvis elevated 4–5 in. In four or five pellets were collected using 2 × 3mm forceps. For BAL fluid collection, a pediatric feeding tube was inserted down into the trachea through direct visualization with a laryngoscope and further into the right or left bronchus at the level of the carina. Ten milliliters of saline were instilled into the bronchus and immediately withdrawn and repeated a maximum of three times until a total of 12–15 ml BAL fluid was retrieved. This procedure generally gave rise to fluid compositions and volumes that were comparable to the bronchoscope-guided BAL (data not shown). Animals were anesthetized with 10 mg/kg ketamine HCL (Fort Dodge Animal Health) prior to all procedures.

L. monocytogenes strains and infection
The attenuated L. monocytogenes strain Listeria ΔactA prfA* (26) was obtained from Nancy Freitag (University of Illinois at Chicago) as previously described (26). Attenuated bacteria were stored in aliquots at −80°C in 20% glycerol (Fisher Chemical, Fairlawn, NJ). Bacteria were washed twice with brain heart infusion broth (BD Bioscience, Franklin Lakes, NJ), and resuspended in 1 ml sterile PBS (Life Technologies, Invitrogen, Carlsbad, CA) for infection.

Isolation of lymphocytes from peripheral blood, BAL fluid, and rectal mucosa
PBLs were isolated from freshly collected EDTA blood by Ficoll-Paque Plus (Amersham, Piscataway, NJ) density gradient centrifugation before analysis. Lymphocytes from BAL fluid and rectal mucosa were isolated as previously described (7).

Immunofluorescent staining and flow cytometric analysis
Cell staining was performed as previously described (28). For cell-surface staining, PBLs were stained with up to five Abs (conjugated to FITC, PE, allophycocyanin, Pacific blue, and PE-Cy7) for 15 min. After staining, cells were fixed with 2% formaldehyde–PBS prior to analysis on a CyAn ADP flow cytometer (DakoCytomation, Carpinteria, CA). Lymphocytes were gated based on forward and side scatter, and pulse width and at least 40,000 gated events were generally analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

Statistical analysis
Statistical analysis was performed using paired two-tailed Student t test or non-parametric test (6) using GraphPad Prism software (GraphPad, La Jolla, CA).

Results

In vitro, it remains unknown whether active L. monocytogenes infection of humans could induce expansion or recall expansion of HMBPP-specific Vγ2Vδ2 T cells. As a proof-of-concept study, two groups of nine rhesus macaques were infected and reinfected systemically with attenuated Listeria ΔactA prfA* (26), respectively. The justification or rationale for inoculating macaques with attenuated Listeria ΔactA prfA* by a systemic route was to induce
subclinical systemic *L. monocytogenes* infection that mimics bacteremia or dissemination of *Listeria* infection in humans. Oral *Listeria* infection does not consistently induce a high rate of systemic *Listeria* infection in immune-competent humans and macaques. Our systemic infection model would allow us to prove an immunology concept, rather than disease pathogenesis, and optimally to examine in vivo immune responses of Vγ2Vδ2 T cells during systemic infection. Although systemic inoculation with wild-type *Listeria* would similarly stimulate macaque γδ T cells, such virulent systemic infection might lead to rapid death, leaving little or no time optimally to study Vγ2Vδ2 T cells. In fact, systemic infection of mice with *L. monocytogenes* was also used to study αβ T cell immune responses (21). We presumed that subclinical systemic *Listeria ΔactA prfA* infection in macaques would induce Vγ2Vδ2 T cell responses that are somehow relevant to those in human systemic listeriosis or septicemia after *L. monocytogenes* infection (25, 31, 32).

The first group of two macaques was inoculated i.m. with 10⁸ CFU *Listeria ΔactA prfA* at day 0 and, at day 35, inoculated again i.v. with 10⁸ CFU *Listeria ΔactA prfA*. The first *L. monocytogenes* inoculation induced detectable increases in percentage and absolute numbers of Vγ2Vδ2 T cells; the secondary inoculation or immunization led to 5- to 10-fold greater expansion of Vγ2Vδ2 T cells that was sustained for at least 3–4 wk (Fig. 1A–C). This initial finding suggested that subclinical systemic *L. monocytogenes* infection could induce an adaptive or recall-like response of Vγ2Vδ2 T cells. Given the possibility that initial low-dose infection could drive optimal recall-like responses of Vγ2Vδ2 T cells in subsequent microbial exposure (8), we infected the second group of seven macaques by inoculating 10⁶ CFU *Listeria ΔactA prfA* i.v. at day 0 and 10⁸ CFU i.v. at day 49, respectively. Notably, the second group of macaques exhibited much greater recall-like expansion of Vγ2Vδ2 T cells after the second exposure to *Listeria ΔactA prfA*, although the primary exposure to the *L. monocytogenes* elicited low-level increases in these γδ T cells (Fig. 1A–C). One week after the second exposure to *Listeria ΔactA prfA*, Vγ2Vδ2 T cells proliferated and expanded from <2% baseline up to 63% of total CD3⁺ T cells, or absolute numbers from <50 cells/µL up to ~13,000 cells/µL of blood (Fig. 1A–C).

Initial expansion and recall-like expansion of Vγ2Vδ2 T cells were driven by *Listeria ΔactA prfA* bacterial burdens as *L. monocytogenes* bacteria were isolated from the blood of the macaques at 1 wk after initial and secondary inoculations with *Listeria ΔactA prfA*, respectively (Fig. 1D). Notably, despite a reduced level of *L. monocytogenes* bacteremia after the *L. monocytogenes* reinfection, Vγ2Vδ2 T cells mounted remarkable recall-like expansion, suggesting that host factors help to adapt.
biopsies and analyzed percentage numbers of Vγ2Vδ2 T cells from rectal mucosal biopsy to isolate lymphocytes from the total mucosal T cells as we previously did (28). Similarly, we determined if Vγ2Vδ2 T cells could traffic to and accumulate in the subsequent resolutions of L. monocytogenes infections each time after i.v. L. monocytogenes inoculation (Fig. 1). Thus, transient subclinical systemic infections with L. monocytogenes could uncover the ability of HMBPP-specific Vγ2Vδ2 T cells to mount adaptive or recall-like immune responses.

Expanded Vγ2Vδ2 T cells during primary and secondary Listeria ΔactA prfA* infections could traffic to and accumulate in the pulmonary compartment and intestinal mucosa

In the setting of Listeria dissemination, bacteremia/septicemia and local mucosal infection likely coexist. We therefore sought to determine if Vγ2Vδ2 T cells activated by subclinical systemic L. monocytogenes infections were able to traffic to mucosa/tissue sites for potential mucosal immunity (7). For simplicity in testing the trafficking function of Vγ2Vδ2 T cells, we compared Vγ2Vδ2 T cells between the rectal and bronchoalveolar interfaces. Consistent with major expansion of Vγ2Vδ2 T cells in the blood, up to 10-fold increases in numbers of Vγ2Vδ2 T cells were detected in BAL fluid after both the primary and secondary inoculations with L. monocytogenes in both groups compared with controls (p = 0.0067 for baseline versus 2 wk after primary infection; p = 0.0024 for baseline versus 1–2 wk after secondary infection; Fig. 2D). In particular, greater increases in Vγ2Vδ2 T cells in BAL fluid from the group 2 macaques were seen after the secondary L. monocytogenes inoculation (Fig. 2D). These results implicated that Vγ2Vδ2 T cells underwent airway trafficking after cellular expansion during L. monocytogenes infections. To examine if Vγ2Vδ2 T cells could traffic to intestinal mucosa, we performed rectal mucosal biopsy to isolate lymphocytes from the biopsies and analyzed percentage numbers of Vγ2Vδ2 T cells in total mucosal T cells as we previously did (28). Similarly, we found ~10-fold increases in numbers of Vγ2Vδ2 T cells in the rectal mucosa after primary and secondary Listeria ΔactA prfA* infections in comparison with controls (p = 0.0288, baseline versus 1–2 wk after secondary infection; Fig. 2D). These results therefore demonstrated that expanded Vγ2Vδ2 T cells during primary and secondary Listeria ΔactA prfA* infections could traffic to and accumulate in the pulmonary compartment and intestinal mucosa.
Expanded Vγ2Vδ2 T cells could evolve into the effector cells producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after subclinical systemic L. monocytogenes infection

We next assessed Vγ2Vδ2 T cells for effector functions producing anti-microbial cytokines over time during primary and secondary Listeria Δact prfA* infections using mAbs against IFN-γ, TNF-α, IL-17/IL-22, IL-4, and perforin for ICS. Whereas Listeria Δact prfA* infections induced very few Vγ2Vδ2 T cells producing IL-22 (data not shown), there were appreciable numbers of Vγ2Vδ2 T effector cells ex vivo producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after secondary L. monocytogenes infection (Figs. 3, 4). In particular, mean absolute numbers of Vγ2Vδ2 T cells producing IFN-γ or IL-4 ex vivo increased up to 1000-fold and 20-fold, respectively, after the second L. monocytogenes exposure (Fig. 4; p = 0.0291 for IFN-γ, p = 0.0096 for IL-4 in comparison between days 59–63 and day 0 or day 42). Vγ2Vδ2 T cells producing IL-17 or perforin increased from baseline <10 cells/µl up to 350 cells/µl and 400 cells/µl of blood, respectively, after the second L. monocytogenes infection (Fig. 4; p = 0.0096 for IL-17, and p = 0.0206 for perforin). Vγ2Vδ2 T cells producing TNF-α increased up to 2000 cells/µl at days 59–63 (Fig. 4, p = 0.0043 in comparisons with day 42 or day 0). These results suggested that expanded Vγ2Vδ2 T cells could evolve into the effector cells producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after L. monocytogenes exposures.

Some Vγ2Vδ2 T cells could exhibit multiple effector functions coproducing IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin during subclinical systemic L. monocytogenes infection

Although effector CD4+ T cells appear to have immune plasticity or flexibility producing or coproducing different cytokines (33), it is unknown whether in vivo-expanded Vγ2Vδ2 T cells can coproduce different cytokines de novo at some points during infections (7–9, 34, 35). As an initial study to explore this, we assessed Vγ2Vδ2 T cells for effector functions coproducing multiple cytokines that have different roles in anti-microbial responses over time after subclinical systemic L. monocytogenes infection. Notably, some Vγ2Vδ2 T cells were able simultaneously to coproduce IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin during subclinical systemic L. monocytogenes infection (Figs. 3, 5). At 1–2 wk after re-exposure to L. monocytogenes, there were significant increases in numbers of Vγ2Vδ2 T cells coproducing IFN-γ and IL-4 (p = 0.0026) or IFN-γ and IL-17 (p = 0.0099) in response to in vitro HMBPP stimulation (Figs. 3, 5). Vγ2Vδ2 T cells capable of in vitro coproducing TNF-α and perforin also increased significantly (p = 0.0017) after the second exposure to L. monocytogenes (Fig. 5).

We then examined if these cytokine coproducers were also detected in the setting without in vitro phosphoantigen HMBPP stimulation. Our recent studies demonstrated that the direct ICS

FIGURE 4. Vγ2Vδ2 T cells evolved into multi-effector functions producing various cytokines after Listeria Δact prfA* infection. (A) Increases in absolute numbers of effector Vγ2Vδ2 T cells producing IFN-γ in group 1 (left) and group 2 (right) of macaques after primary L. monocytogenes infection and L. monocytogenes reinfection, p = 0.0291 in comparison between days 59/63 and day 0 or day 42. Vγ2Vδ2 T cells producing TNF-α increased up to 2000 cells/µl at days 59–63 (Fig. 4, p = 0.0043 in comparisons with day 42 or day 0). These results suggested that expanded Vγ2Vδ2 T cells could evolve into the effector cells producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after L. monocytogenes exposures.

(A) Increase in absolute numbers of effector Vγ2Vδ2 T cells producing IFN-γ in group 1 (left) and group 2 (right) of macaques after primary L. monocytogenes infection and L. monocytogenes reinfection, p = 0.0291 in comparison between days 59/63 and day 0 or day 42. Vγ2Vδ2 T cells producing TNF-α increased up to 2000 cells/µl at days 59–63 (Fig. 4, p = 0.0043 in comparisons with day 42 or day 0). These results suggested that expanded Vγ2Vδ2 T cells could evolve into the effector cells producing IFN-γ, TNF-α, IL-4, IL-17, or perforin after L. monocytogenes exposures.
assay without in vitro Ag stimulation was useful for direct detection of effector T cells producing cytokines IFN-γ, IL-17/IL-22, TNF-α, or perforin de novo, as the direct ICS analysis could readily detect those evolving effector T cells over time during *M. tuberculosis* or malaria infection of macaques, but not during longitudinal follow-up of control uninfected individuals (36, 37). The direct ICS analysis showed that at 1–2 wk after re-exposure to *L. monocytogenes*, there were significant increases in effector Vγ2Vδ2 T cells that could coproduce IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin de novo without the need for in vitro HMBPP stimulation (Fig. 5, p < 0.01 for all three sets of coproducers). Thus, some Vγ2Vδ2 T cells could exhibit multiple effector functions coproducing IFN-γ/IL-4, IFN-γ/IL-17, or TNF-α/perforin during subclinical systemic *L. monocytogenes* infection. To our knowledge, this is the first demonstration of multifunctional responses of Vγ2Vδ2 T cells during microbial infection of macaques.

*In vivo-expanded Vγ2Vδ2 T cells during subclinical systemic L. monocytogenes infection could directly lyse L. monocytogenes-infected target cells and inhibit intracellular L. monocytogenes bacteria*  

Emergence of Vγ2Vδ2 T cells producing perforin and other cytokines de novo raised a question as to whether these in vivo-expanded γδ T effector cells were able directly to lyse *L. monocytogenes*-infected target cells and inhibit intracellular *L. monocytogenes* bacteria. To test Vγ2Vδ2 T cell-mediated lysis of *L. monocytogenes*-infected target cells, we used a cytotoxicity assay testing the ability of Vγ2Vδ2 T cells directly to lyse *L. monocytogenes*-infected autologous target cells without in vitro stimulation of these γδ T cells. Because *Listeria* ΔactA prfA* infections exclusively expanded Vγ2Vδ2 T cells but not other γδ T subsets in blood, we purified in vivo-expanded Vγ2Vδ2 T cells directly from PBMCs after the infection using anti-Vγ2 mAb and immunobeads (MACS) as previously described (38). Purified Vγ2Vδ2 T cells were then cocultured with *L. monocytogenes*-infected autologous monocyte-derived DCs and assessed for CTL killing of DCs. Vγ2Vδ2 T cells that subtly expanded at day 15 after *L. monocytogenes* infection (Fig. 1) did not mediate apparent CTL killing of DCs. Vγ2Vδ2 T cells but not other γδ T subsets infected *L. monocytogenes*-infected DCs (Fig. 6A). Virtually, these Vγ2Vδ2 T cells isolated at peak expansion were able to lyse almost 50% of *L. monocytogenes*-infected DCs after subtracting the values of various controls (Fig. 6A, 6B). This potent CTL lysis of *L. monocytogenes*-infected DCs appeared to be consistent with the remarkable increases in numbers of Vγ2Vδ2 T
infection could directly lyse \( \text{L. monocytogenes} \) bacteria growth. (A) Representative histograms for flow-based cytotoxicity assay show that \( \text{V} \gamma \text{V} \delta \) T effector cells that expanded in vivo after \( \text{L. monocytogenes} \) reinfection exhibited an increased lysis of \( \text{L. monocytogenes} \)-infected DCs. Data are gated on CD11c+ DC. (B) Bar graphs demonstrated that effector \( \text{V} \gamma \text{V} \delta \) T cells that expanded in vivo after \( \text{L. monocytogenes} \) reinfection exhibited an increased lysis of \( \text{L. monocytogenes} \)-infected DCs. \( n = 5 \). Cell lysis is expressed as percentage number of PI-stained dead cells (“%PI−”) in CD11c+ DCs. Day 59 \( \text{V} \gamma \text{V} \delta \) T cells lysed more \( \text{L. monocytogenes} \)-infected DCs than did day 15 \( \text{V} \gamma \text{V} \delta \) T cells \((p = 0.0003)\) and B cells \((p = 0.0231)\), respectively. Day 59 \( \text{V} \gamma \text{V} \delta \) T cells lysed more \( \text{L. monocytogenes} \)-infected DCs than did control DCs \((p = 0.0057)\), more than did the culture with \( \text{L. monocytogenes} \)-infected DCs only \((p = 0.0005)\), and more than did the culture with control DCs only \((p = 0.0071)\). (C) In vivo-expanded \( \text{V} \gamma \text{V} \delta \) T cells after \( \text{L. monocytogenes} \) reinfection could limit intracellular \( \text{L. monocytogenes} \) bacterial growth in monocyte-derived macrophages. \( n = 4 \), \( p < 0.0001 \) compared with baseline; \( p = 0.001 \) compared with B cells. D, Day; LM, \( \text{L. monocytogenes} \).

To determine if in vivo-expanded \( \text{V} \gamma \text{V} \delta \) T cells could inhibit intracellular \( \text{L. monocytogenes} \) bacteria in the infected cells, we measured CFU counts in lysate derived from \( \text{L. monocytogenes} \)-infected macrophages cocultured with purified \( \text{V} \gamma \text{V} \delta \) T cells. Notably, in vivo-expanded \( \text{V} \gamma \text{V} \delta \) T cells purified at day 63 were able to inhibit intracellular \( \text{L. monocytogenes} \) bacteria more efficiently than those isolated at day 0 \((p < 0.0001)\) when they were cocultured with \( \text{L. monocytogenes} \)-infected macrophages (Fig. 6C). The reduction of \( \text{L. monocytogenes} \) CFU counts by day 63 \( \text{V} \gamma \text{V} \delta \) T cells was significantly more striking than that by the controls, B cells plus \( \text{L. monocytogenes} \)-infected macrophages \((p < 0.001)\), or \( \text{L. monocytogenes} \)-infected macrophages only \((p < 0.0001)\).

Thus, in vivo-expanded \( \text{V} \gamma \text{V} \delta \) T cells after \( \text{Listeria \Delta actA prfA}^{−} \) infection could directly lyse \( \text{L. monocytogenes} \)-infected target cells and inhibit intracellular \( \text{L. monocytogenes} \) bacteria.

**Discussion**

We present the first in vivo study, to our knowledge, to examine \( \text{L. monocytogenes} \)-induced immune responses of HMBPP-specific \( \text{V} \gamma \text{V} \delta \) T cells during primary and secondary subclinical \( \text{L. monocytogenes} \) infections (3). Subclinical systemic \( \text{L. monocytogenes} \) infection of primes provides an interesting setting for studies of in vivo HMBPP-specific \( \text{V} \gamma \text{V} \delta \) T cells in that \( \text{L. monocytogenes} \) is the only pathogenic bacterium known to contain both mevalonate and non-mevalonate pathways of isoprenoid biosynthesis, concurrently producing metabolites such as HMBPP and IPP (3). Our findings in \( \text{L. monocytogenes} \)-infected macaques appear to be novel, as \( \text{L. monocytogenes} \) bacterial infections induce multieffector-functional immune responses of HMBPP-specific \( \text{V} \gamma \text{V} \delta \) T cells. The multieffector-functional immune responses of \( \text{V} \gamma \text{V} \delta \) T cells are characterized by the remarkable recall-like expansion, pulmonary or mucosal trafficking, broad effector functions producing or coproducing Th1 and Th2 or Th17 cytokines, direct lysis of \( \text{L. monocytogenes} \)-infected target cells, and inhibition of intracellular \( \text{L. monocytogenes} \) bacteria. To our knowledge, our work represents one of the significant immunological studies that elucidate broad anti-microbial effector functions of HMBPP-specific \( \text{V} \gamma \text{V} \delta \) T cells in vaccination or subclinical infection.

One of the interesting immune features for \( \text{V} \gamma \text{V} \delta \) T cells during subclinical systemic \( \text{L. monocytogenes} \) infections is their ability to mount remarkable recall-like expansion. Clearly, absolute numbers of \( \text{V} \gamma \text{V} \delta \) T cells can increase from <50 cells/µL up to 15,000 cells/µL within a week after secondary \( \text{L. monocytogenes} \) inoculation. This extraordinary recall-like expansion is consistent with the adaptive immune response seen in macaques reinfeeted with \( \text{Mycobacterium bovis} \) BCG or those animals infected with \( \text{M. bovis} \) BCG and then reinfeeted with \( \text{M. tuberculosis} \) (6). These three bacterial pathogens share a common ability to produce HMBPP and to infect monocytes/macrophages/DCs intracellularly. However, in both BCG reinfection and \( \text{Listeria} \)}
reinfecion, bacteria isolated from the blood are much lower than the bacteremia during primary infections, but the lower bacterial burdens induce much faster, greater, and longer expansion of Vγ2Vδ2 T cells, suggesting that host factors also play a role in mounting remarkable recall-like expansion. Our results are also consistent with the scenario that primary infection with HMBPP-producing microbes could optimally prime Vγ2Vδ2 T cells and such primed γδ T cells could mount remarkable recall-like expansion in response to subsequent reinfection. Data from human studies (39–45) also support the notion that human Vγ2Vδ2 T cells can mount adaptive or recall-like immune response in infections. Furthermore, our findings suggest that in vivo Vγ2Vδ2 T cell responses to intracellular infectious agents may be different from the rapid Vγ2Vδ2 T cell expansion after repeated phosphoantigen/IL-2 cotreatments (7, 34).

Subclinical systemic L. monocytogenes infections also uncover pulmonary or intestinal mucosal migration characteristics of HMBPP-specific Vγ2Vδ2 T cells. This functional aspect is generally considered important for mucosal/pulmonary immunity as most microbial pathogens invade hosts at the mucosal interface. Vγ2Vδ2 T cells appear to emerge as a dominant T cell subpopulation among the T cell pool in airway and rectal mucosa after Listeria infections, as they compose up to 30% of total CD3+ T cells in those mucosae (Fig. 2). Given the possibility that lymphoid tissues in small intestines are more enriched than in rectal mucosa, we anticipate that Vγ2Vδ2 T cells may more apparently traffic to and accumulate in the small intestinal mucosa. The trafficking and accumulation of Vγ2Vδ2 T cells in intestinal mucosae might underscore the importance of these cells for contributing to mucosal immunity against food-borne L. monocytogenes infection. We previously demonstrated that expanded Vγ2Vδ2 T cells during BCG or M. tuberculosis infections or after HMBPP/IL-2 treatments could similarly undergo pulmonary/mucosal trafficking and accumulation (6, 7, 13, 46). Thus, pulmonary and intestinal mucosal trafficking/accumulations appear to be a common biological feature for activated Vγ2Vδ2 T cells regardless of the in vivo stimuli.

Whereas a number of studies are focused on innate production of cytokines by Vγ2Vδ2 T cells (4, 7, 34), the current longitudinal follow-up studies reveal broad effector functions producing or coproducing cytokines by Vγ2Vδ2 T cells during adaptive or recall-like immune response in L. monocytogenes infections. L. monocytogenes-expanded Vγ2Vδ2 T cells are able to produce IFN-γ, TNF-α, IL-4, IL-17, and perforin, and some of them can simultaneously coproduce IL-17 and IFN-γ, IL-4 and IFN-γ, or TNF-α and perforin at peak expansion time after L. monocytogenes reinfection. It is noteworthy that such coproduction of cytokines can even be detected by direct ICS without prior in vitro HMBPP stimulation, which is close to an in vivo effector function for spontaneous cytokine production. Simultaneous coproduction of these cytokines by Th cells appears to be an uncommon, but not impossible, immune event as the development for each of the Th subsets producing Th1, Th2, and Th17 cytokines is controlled tightly by individual unique master transcriptional factors such as T-bet, GATA-3, and RORγT (33). Recent studies of αβ T cells have shown that IFN-γ can regulate production of IL-17 (47), and IFN-γ and IL-4 may be coproduced in some instances (48). During L. monocytogenes infection, production or coproduction of Th1, Th2, and Th17 cytokines by Vγ2Vδ2 T effector cells may play a role in activation of a variety of other immune cells such as DCs/macrophages, CD4+CD8α T cells, and B cells and therefore may bridge innate and adaptive immunity. In addition, IFN-γ and IL-17A can contribute to immunity to Listeria infection (20, 49), and perforin is protective against L. monocytogenes infection in mice (50).

Another novel finding from the current study is the demonstration of direct lysis of Listeria-infected target cells by in vivo-expanded Vγ2Vδ2 T cells without in vitro HMBPP stimulation. Early studies showed that extensive in vitro activation/stimulation of Vγ2Vδ2 T cells by phosphoantigen/pathogen and IL-2 allows the cultured Vγ2Vδ2 T cells to lyse Mycobacterium-infected cells or inhibit intracellular mycobacteria (11, 12, 51). In the current study, we demonstrate that in vivo-expanded Vγ2Vδ2 T cells after systemic re-exposure to L. monocytogenes can directly lyse L. monocytogenes-infected CD11c+ DCs without the need for in vitro Ag/cytokine activation. This novel finding appears to be linked to the perforin-producing effector function of Vγ2Vδ2 T cells, as in vivo-expanded Vγ2Vδ2 T cells increase after the second infection and produce perforin de novo even without the need for in vitro HMBPP stimulation. The direct lysis might also be explained by the fact that most Vγ2Vδ2 T cells after the second infection express effector phenotype (data not shown). Importantly, these in vivo-expanded Vγ2Vδ2 T cells can inhibit intracellular L. monocytogenes bacterial replication. To our knowledge, this is the first experimental evidence showing direct lysis of microbe-infected cells and inhibition of intracellular L. monocytogenes by in vivo-expanded Vγ2Vδ2 T cells without the prior in vitro Ag stimulation.

Thus, we have demonstrated multifunctional functions of HMBPP-specific Vγ2Vδ2 T cells in immune responses to systemic exposures to L. monocytogenes. Results from comprehensive analyses of HMBPP-specific Vγ2Vδ2 T cells in the current study now provide a rationale to conduct comparative studies in the future using mutants of L. monocytogenes that fail to produce HMBPP. Our findings in the current study also raise the possibility to conduct further in vivo studies and investigate the role of Vγ2Vδ2 T cells in controlling L. monocytogenes infection.

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Disclosures
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References


