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Multieffector-Functional Immune Responses of HMBPP-Specific Vγ2Vδ2 T Cells in Nonhuman 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 nonmevalonate isoprenoid biosynthesis pathways that produce metabolic 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: 1285–1293.

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-3-methyl-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, nonmevalonate pathway (2, 3). IPP is an intermediate metabolite found in both pathways, whereas HMBPP is only produced in the nonmevalonate 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 (6). Furthermore, major expansion of Vγ2Vδ2 T effector cells after HMBPP plus IL-2 post-challenge treatment can lead to homeostatic protection against severe pneumonia 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 nonmevalonate 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, nonhuman primate models appear to provide a unique model system in which to explore immune responses and
effector function of HMBPP-specific V\textsubscript{y}2V\textsubscript{82} T cells during \textit{L. monocytogenes} infection. In the current study, rhesus macaques were infected systemically with an attenuated \textit{L. monocytogenes} strain (\textit{Listeria} \textit{\Delta ac\textsubscript{t}a prfA\textsuperscript{a}}), which carries a deletion of the \textit{actA} gene and a mutation of the constitutively active \textit{prfA} gene (26, 27). The \textit{actA} deletion abrogates the ability of \textit{L. monocytogenes} to polymerize actin and to mediate cell-to-cell spread of \textit{L. monocytogenes}, although the \textit{prfA} mutation upregulates \textit{L. monocytogenes} gene expression (26). The subclinical systemic \textit{Listeria \Delta ac\textsubscript{t}a prfA\textsuperscript{a}} infection in macaques allowed us optimally to demonstrate multieffector-functional immune responses of V\textsubscript{y}2V\textsubscript{82} T cells. \textit{L. monocytogenes}-activated V\textsubscript{y}2V\textsubscript{82} 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-\gamma, IL-4 and IFN-\gamma, or TNF-\alpha and perforin. The in vivo-expanded V\textsubscript{y}2V\textsubscript{82} T cells can directly lyse \textit{L. monocytogenes}-infected target cells and inhibit intracellular \textit{L. monocytogenes} bacteria without the need for prior in vitro phosphoantigen 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 \textit{10}\textsuperscript{9} CFU i.m. attenuated \textit{Listeria \Delta ac\textsubscript{t}a prfA\textsuperscript{a}} (26) i.m. and boosted on day 35 with \textit{10}\textsuperscript{8} CFU i.v. Seven macaques were infected at day 0 with \textit{10}\textsuperscript{9} CFU i.v. and boosted on week 7 with \textit{10}\textsuperscript{8} 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 and the rectum was examined using 3 mm 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 bronchoesophageal BAL fluid (data not shown). Animals were anesthetized with 10 mg/kg ketamine HCL (Fort Dodge Animal Health) prior to all procedures.

\textit{L. monocytogenes} strains and infection

The attenuated \textit{L. monocytogenes} strain \textit{Listeria \Delta ac\textsubscript{t}a prfA\textsuperscript{a}} (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, 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 injection.

Isolation of lymphocytes from peripheral blood, BAL fluid, and rectal mucosae

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 mucosae 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. To generate \textit{L. monocytogenes}-infected targets, monocytes isolated from PBMCs were cultured for 3–4 d to generate monolayer macrophages. Monocyte-derived macrophages were incubated for 90 min with \textit{10}\textsuperscript{7} \textit{L. monocytogenes} bacteria (multiplicity of infection = 100), then incubated in RPMI 1640 media containing 10% FBS supplemented with gentamicin for 2 h to kill extracellular bacteria and subsequently washed twice with RPMI with 10% FBS. Target cells (\textit{10}\textsuperscript{7}) were cocultured with \textit{10}\textsuperscript{7} purified autologous effector V\textsubscript{y}2V\textsubscript{82} T cells or control CD20\textsuperscript{+} B cells in each well (performed in triplicate) of a 96-well plate. After overnight coculture, plates were centrifuged to pellet cells, supernatant was removed, and cells were incubated in 200 μl sterile water to lyse target cells and release \textit{L. monocytogenes} bacteria from infected cells. Ten-fold serial dilutions of lysis were made in sterile water and plated on BHI plates containing streptomycin to select for \textit{L. monocytogenes} bacteria (30).

Statistical analysis

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

Results

Transient subclinical systemic \textit{L. monocytogenes} infections uncovered the ability of HMBPP-specific V\textsubscript{y}2V\textsubscript{82} T cells to mount adaptive or recall-like immune responses

Whereas it is well known that \textit{L. monocytogenes} bacteria can produce large amounts of HMBPP activating V\textsubscript{y}2V\textsubscript{82} T cells in vitro, it remains unknown whether active \textit{L. monocytogenes} infection of humans could induce expansion or recall expansion of HMBPP-specific V\textsubscript{y}2V\textsubscript{82} T cells. As a proof-of-concept study, two groups of nine rhesus macaques were infected and reinjected systemically with attenuated \textit{Listeria \Delta ac\textsubscript{t}a prfA\textsuperscript{a}} (26), respectively. The justification or rationale for inoculating macaques with attenuated \textit{Listeria \Delta ac\textsubscript{t}a prfA\textsuperscript{a}} 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 γβ 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 septicaemia 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 10⁵ CFU *Listeria* ΔactA prfA* i.v. at day 0 and 10⁶ 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

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**FIGURE 1.** Transient systemic *L. monocytogenes* infections uncovered the ability of HMBPP-specific Vγ2Vδ2 T cells to mount adaptive or recall-like immune responses. (A) Representative histograms showing Vγ2Vδ2 T cells in PBMCs on day 0 (top) and at the peak of Vγ2Vδ2 T cell expansion during subclinical systemic *L. monocytogenes* infection. Data are gated on CD3⁺ lymphocytes. (B) Vγ2Vδ2 T cells, expressed as percent of CD3⁺ T cells, demonstrated expansion and recall-like expansion after primary and secondary *L. monocytogenes* infections, respectively, in group 1 (left) and in group 2 (right). Two vertical dotted lines denote the time points for the first and second *L. monocytogenes* inoculations, respectively. (C) Increases in absolute numbers of Vγ2Vδ2 T cells after primary and secondary *L. monocytogenes* infections. *p* < 0.0001 for comparisons between day 0 and day 56 or between day 0 and days 59/63. (D) Expansion and recall-like expansion of Vγ2Vδ2 T cells were associated with subsequent transient *L. monocytogenes* bacteremia. Shown are *L. monocytogenes* CFU counts in the blood of rhesus macaques after initial *L. monocytogenes* infection (day 0) or *L. monocytogenes* reinfection (day 49) by i.v. route (*n* = 4). D, Day.
recall-like immune response (Fig. 1D). Notably, initial expansion and recall-like expansion of Vγ2Vδ2 T cells were associated with the subsequent resolutions of L. monocytogenes bacteremia 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. 2). 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. 2). 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 mucosae, 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 mucosae 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 IL-17 after 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.
**FIGURE 5.** *Listeria ΔactA prfA*+ infection induced multieffector Vγ2Vδ2 T cells coproducing different cytokines in the presence (right) or absence (left) of HMBPP stimulation. (A) Increases in absolute numbers of Vγ2Vδ2 T cells coproducing IL-17 and IFN-γ after *L. monocytogenes* infection. *p* < 0.01 for the setting with (right) or without (left) the need for HMBPP stimulation (see the table at the bottom of the figure). (B) Increases in absolute numbers of Vγ2Vδ2 T cells coproducing IL-17 and IFN-γ after *L. monocytogenes* infection. *p* < 0.01 for the setting with (right) or without (left) the need for HMBPP stimulation (see the table at the bottom of the figure). (C) Increases in absolute numbers of Vγ2Vδ2 T cells coproducing perforin and TNF-α after *L. monocytogenes* infection. *p* < 0.01 for the setting with (right) or without (left) the need for HMBPP stimulation (see the table at the bottom of the figure). D, Day.

In vivo-expanded Vγ2Vδ2 T 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 immuno-MACS (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 *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 cells exclusively expanded 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 multieffector-functional 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 immuno-MACS (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 *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 cells exclusively expanded 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 multieffector-functional responses of Vγ2Vδ2 T cells during microbial infection of macaques.

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compared with B cells. D, Day; LM, L. monocytogenes could limit intracellular L. monocytogenes (T cells lysed more T cells lysed more L. monocytogenes (controls, B cells plus L. monocytogenes target cells and inhibit intracellular infection could directly lyse prfA* (V L. monocytogenes 6C). The reduction of cocultured with L. monocytogenes efficiently than those isolated at day 0 (p = 0.0005), and more than did the culture with L. monocytogenes-infected DCs only (p = 0.0005), and more than did the culture with control DCs only (p = 0.0071). In vivo-expanded Vγ2Vδ2 T cells after L. monocytogenes reinfection could limit intracellular 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, L. monocytogenes. effector cells producing perforin de novo at 1–2 wk after re-exposure to L. monocytogenes (Figs. 4D, 5).

To determine if in vivo-expanded Vγ2Vδ2 T cells could inhibit intracellular L. monocytogenes bacteria in the infected cells, we measured CFU counts in lysate derived from L. monocytogenes-infected macrophages cocultured with purified Vγ2Vδ2 T cells. Notably, in vivo-expanded Vγ2Vδ2 T cells purified at day 63 were able to inhibit intracellular L. monocytogenes bacteria more efficiently than those isolated at day 0 (p < 0.0001) when they were cocultured with L. monocytogenes-infected macrophages (Fig. 6C). The reduction of L. monocytogenes CFU counts by day 63 Vγ2Vδ2 T cells was significantly more striking than that by the controls, B cells plus L. monocytogenes-infected macrophages (p < 0.001), or L. monocytogenes-infected macrophages only (p < 0.0001).

Thus, in vivo-expanded Vγ2Vδ2 T cells after Listeria ΔactA prfA* infection could directly lyse L. monocytogenes-infected target cells and inhibit intracellular L. monocytogenes bacteria.

Discussion

We present the first in vivo study, to our knowledge, to examine L. monocytogenes-induced immune responses of HBMP-specific Vγ2Vδ2 T cells during primary and secondary subclinical L. monocytogenes infections (3). Subclinical systemic L. monocytogenes infection of primates provides an interesting setting for studies of in vivo HBMP-specific Vγ2Vδ2 T cells in that L. monocytogenes is the only pathogenic bacterium known to contain both mevalonate and nonmevalonate pathways of isoprenoid biosynthesis, concurrently producing metabolites such as HBMP and IPP (3). Our findings in L. monocytogenes-infected macaques appear to be novel, as L. monocytogenes bacterial infections induce multieffector-functional immune responses of HBMP-specific Vγ2Vδ2 T cells. The multieffector-functional immune responses of Vγ2Vδ2 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 L. monocytogenes-infected target cells, and inhibition of intracellular L. monocytogenes bacteria. To our knowledge, our work represents one of the significant immunological studies that elucidate broad anti-microbial effector functions of HBMP-specific Vγ2Vδ2 T cells in vaccination or subclinical infection.

One of the interesting immune features for Vγ2Vδ2 T cells during subclinical systemic L. monocytogenes infections is their ability to mount remarkable recall-like expansion. Clearly, absolute numbers of Vγ2Vδ2 T cells can increase from <50 cells/μl up to 15,000 cells/μl within a week after secondary L. monocytogenes inoculation. This extraordinary recall-like expansion is consistent with the adaptive immune response seen in macaques reinfected with Mycobacterium bovis BCG or those animals infected with M. bovis BCG and then reinfected by M. tuberculosis (6). These three bacterial pathogens share a common ability to produce HBMP and to infect monocytes/macrophages/DCs intracellularly. However, in both BCG reinfection and Listeria
reinfection, 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 responses to intracellular infectious agents. Moreover, we have shown that IFN-γ and perforin at peak expansion time after L. monocytogenes 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.

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Disclosures
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