Molecular Medicine

Human γδ T Cells Augment Antigen Presentation in *Listeria Monocytogenes* Infection

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Circulating γδ T cells in healthy individuals rapidly respond to bacterial and viral pathogens. Many studies have demonstrated that γδ T cells are activated and expanded by *Listeria monocytogenes* (*L. monocytogenes*), a foodborne bacterial pathogen with high fatality rates. However, the roles of γδ T cells during *L. monocytogenes* infection are not clear. In the present study, we characterized the morphological characteristics of phagocytosis in γδ T cells after *L. monocytogenes* infection using transmission electron microscopy. Results show activation markers including human leucocyte antigen DR (HLA–DR) and lymph node–homing receptor CCR7 on γδ T cells were upregulated after stimulation via *L. monocytogenes*. Significant proliferation and differentiation of primary αβ T cells was also observed after coculture of peripheral blood mononuclear cells with γδ T cells anteriorly stimulated by *L. monocytogenes*. *L. monocytogenes* infection decreased the percentage of γδ T cells in mouse intraepithelial lymphocytes (IELs) and increased MHC-II expression on the surface of γδ T cells *in vivo*. Our findings shed light on antigen presentation of γδ T cells during *L. monocytogenes* infection.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00214

INTRODUCTION

Human γδ T cells are a subset of T cells with a T cell receptor (TCR) composed of γ and δ chains (1). They constitute a small proportion (3~10%) of circulating $CD3^+$ T-lymphocytes in peripheral blood. Compared with αβ T cells, γδ T cells recognize antigens without major histocompatibility complex (MHC) restriction and without help from antigen presenting cells (APC). They directly bind to stress-induced ligands such as heat shock proteins and mutS homolog 2 (hMSH2) (2–4). γδ T cells are believed to play import roles in innate antimicrobial and antitumor immunity defense (5). In addition to directly binding stress-induced ligand and killing target cells, γδ T cells also serve as APCs to elicit subsequent specific immune responses (6,7). Brandes *et al.* showed that activated human γδ T cells present protein antigens to naïve $CD4^+$ and $CD8^+$ αβ T cells (8,9). Wu *et al.* found that naïve peripheral blood γδ T cells phagocytized IgG opsonized *Escherichia coli* (*E. coli*), IgG opsonized latex beads and whole influenza A virus matrix (M1) protein, which produced subsequent functional effects (10).

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive, intracellular bacterium that causes listeriosis, primarily affecting immunocompromised individuals, pregnant women and newborns. It is the only pathogenic bacterium known to contain both mevalonate and nonmevalonate pathways of isoprenoid biosynthesis, concurrently producing metabolites such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and isopentenyl pyrophosphate (IPP) (11) which are the specific ligands of γδ TCR (12,13). Clinical experiments have confirmed that γδ T cells are overrepresented in the blood of patients during *L. monocytogenes* infections by up to 50% of total T cells (14). The expanded γδ T cells produce IFN-γ, TNF-α, IL-4, IL-17 or perforin to mediate inflammation or lyse *L. monocytogenes*-infected target cells directly (15). They also regulate the chemokine production in macrophages (16). However, it

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Submitted September 29, 2015; Accepted for Publication September 16, 2016; Published Online (www.molmed.org) September 19, 2016.

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is unknown whether γδ T cells serve as APCs during *L. monocytogenes* infection. We hypothesized that they uptake *L. monocytogenes* and process and present antigens to αβ T cells to induce specific adaptive immune responses. It is fascinating to think that γδ T cells may internalize antigens in a phagocytizing manner like phagocytes, which has been ignored for some time. Our findings from an *in vitro* experimental system prove that γδ T cells have an internalizing capability when bound to *L. monocytogenes* and induce a specific immune response to *L. monocytogenes*. This indicates that γδ T cells serve as APCs during *L. monocytogenes* infection.

MATERIALS AND METHODS

Bacteria

Toxicity strain *L. monocytogenes* ATCC 19115 (serotype 4b) was a quality control strain purchased from American Type Culture Collection (ATCC). The bacteria were cultured aerobically in brain heart infusion (BHI) at 37°C. BHI broth was obtained from BD-Biosciences.

Human Blood Samples

Peripheral blood samples of healthy adult donors were collected with informed consent. The study was approved by the ethical board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

Purification of Naïve γδ T and αβ T Cells

Peripheral blood mononuclear cells (PBMCs) from peripheral blood samples were separated by density gradient centrifugation using a Ficoll density gradient (GE Healthcare companies) as described previously (17,18). Naïve γδ T and αβ T cells were enriched from PBMCs by high-gradient magnetic cell separation (MACS) according to the manufacturer's instructions (Miltenyi Biotechnology companies). The purity of γδ T and αβ T cells were above 90% and 95%, respectively, as analyzed by flow cytometry.

Generation of Activated γδ T and αβ T Cells and Rested γδ T Cells

The activation and expansion of γδ T cells was described previously (19,20). Briefly, each well of 24-well plate was coated with 0.5-μg antipan-TCRγδ mAb (Immunotech, Beckman Coulter). After solution was removed, PBMCs were added to the plates and cultured in RPMI 1640 medium (Corning, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL company), 200 IU/mL recombinant human IL-2 (Beijing Read United Cross Pharmaceutical Co., Ltd.), 100 mg/mL penicillin and 100 U/mL streptomycin at 37°C, 5% CO_2 for five days. PBMCs were transferred to culture bottle and passaged based on growth condition until the purity was above 90%. IL-2 was removed for 24 h to obtain rested γδ T cells.

For activated αβ T cells, we followed the instructions of T Cell Activation, In Vitro from eBioscence. The culture plate was coated with 5–10 μg/mL anti-CD3e Ab for 2 h at 37°C. PBMCs were transferred to the plate and added soluble anti-CD28 at 2 μg/mL to the culture medium (RPMI 1640 with 10% FBS, 200 IU/mL IL-2 and penicillin/streptomycin). After incubation for four days, cells were harvested and processed for assays.

Infection with *L. monocytogenes*

L. monocytogenes was cultured in BHI broth for three to five hours, the number of CFU was calculated based on growth curve as described previously (21). Bacteria were washed twice and resuspended in phosphate-buffered saline (PBS). *L. monocytogenes* was added at the desired bacterium-to-cell ratios (ratio = 5 or 50) to γδ T cells, αβ T cells or PBMCs. They were incubated in RPMI 1640 medium with 10% fetal calf serum at 37°C. After one hour or three hours penicillin and gentamicin were added to kill extracellular bacteria.

Coculture Experiment

The infected γδ T cells were cultured with homologous PBMCs or αβ T cells at different ratios (1:1 or 1:10) in RPMI 1640 medium with 10% fetal bovine serum (FBS) and antibiotics at 37°C for six days. To ensure consistency of cells, some freshly isolated γδ T cells from PBMCs were cultured, the remaining were frozen in liquid nitrogen before *L. monocytogenes* infection. The total cell number was approximately 1×10^6/well. After six days in coculture (9), the different group cell numbers were counted and converted to a ratio by comparison with the initial PBMC number.

L. monocytogenes Infection Assay

Female 10–12 wk BALb/c mice were purchased from the Laboratory Animal Research Institute of the Chinese Academy of Medical Sciences. Mice were housed at the animal facilities at the Peking Union Medical College and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences in 2002. After three hours culture, 10^8 CFU Listeria were resuspended in 0.2 mL PBS. Mice were infected by intragastric administration, then killed after 12 h, 24 h, 36 h or 48 h. Intestinal lymphoid cells were isolated by Percoll gradient centrifugation (22). The percentage of γδ T cells and related molecular expression were detected by flow cytometry.

Transmission Electron Microscopy (TEM)

After *L. monocytogenes* infection, γδ T cells or αβ T cells were washed with PBS and fixed in 2.5% gluteraldehyde. Preparation for TEM was performed at the Electron Microscopy Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, as described previously (10). Briefly, after fixation, T cells were soaked and washed three times with 0.1 mol/L PBS before post fixing with 1% osmium tetroxide solution in wash buffer at room temperature (RT) for two hours. Samples were then dehydrated in graded ethanol seven times and embedded in acetone and pure Epon. Ninety nanometer ultrathin sections were stained with 8% uranyl acetate and lead citrate before observation under the electron microscope (JEOL).

Flow Cytometry (FCM)

Samples of 1×10^6 cells were harvested, washed and resuspended in 50 μL of PBS containing 1% BSA. Different fluorochrome-conjugated monoclonal antibodies were added per reaction. After incubation at 4°C for 20 min, cells were washed with PBS, resuspended in 500 μL of PBS containing 1% formaldehyde and analyzed on a BD Accuri C6 Flow Cytometer (18,23). FITC-conjugated anti-TCRγδ, PE-conjugated anti-TCRαβ and the respective isotypic control mAbs were purchased from Immunotech. FITC-conjugated anti-CD4, PE-conjugated anti-CD8a and IL-17A, PE/Cy7-conjugated anti-IL-4 and APC-conjugated anti-IFN-γ were purchased from BD Pharmingen. BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit with BD GolgiPlug was used to detect intracellular cytokines according to the manual. In short, after cell surface antigens were stained, cells were resuspended in fixation/permeabilization solution for 20 min at 4°C. Cells were washed two times in BD Perm/Wash buffer and stained for intracellular cytokines at 4°C for 30 min in the dark. Cells were washed with BD Perm/Wash buffer and resuspended in staining buffer prior to flow cytometry.

Statistical Analysis

Data are expressed as the mean ± standard error of mean (SEM). One-tailed Student *t* test (SPSS version 16.0 software) was used to determine significant differences between groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Human γδ T Cells Possess Phagocytic Capacity

Previous studies suggest γδ T cells possess antigen presenting ability (24,25). To confirm this, we first assessed the phagocytic capacity of γδ T cells. γδ T cells were expanded by culturing PBMCs in antipan-TCRγδ mAb-coated plates with RPMI 1640 medium containing IL-2 for 10 d. The purity of the γδ T cells was assessed by FCM and reached 90 % (Figure 1A1). These IL-2-activated γδ T cells displayed a "hairy" appearance with a large regular round nucleus and thin cytoplasm under TEM (Figure 1B1). However, after incubation with *L. monocytogenes* for one hour, approximately 20% of γδ T cells resembled phagocytic cells. *L. monocytogenes* were surrounded by pseudopod-like protrusions extending from the cytomembrane of some activated γδ T cells (Figure 1B2). Three hours later, γδ T cells showed membrane-bound phagosomal structures containing more *L. monocytogenes* bacteria (Figure 1B3). After longer incubation, we observed many dead γδ T cells with *L. monocytogenes* bacteria (Figure 1B4). When rested by IL-2 withdrawal for 24 h before *L. monocytogenes* incubation, γδ T cells phagocytized the bacterium with the same percentage (Figure 1B5). The morphology of γδ T cells was similar to activated γδ T cells, but the size was slightly smaller.

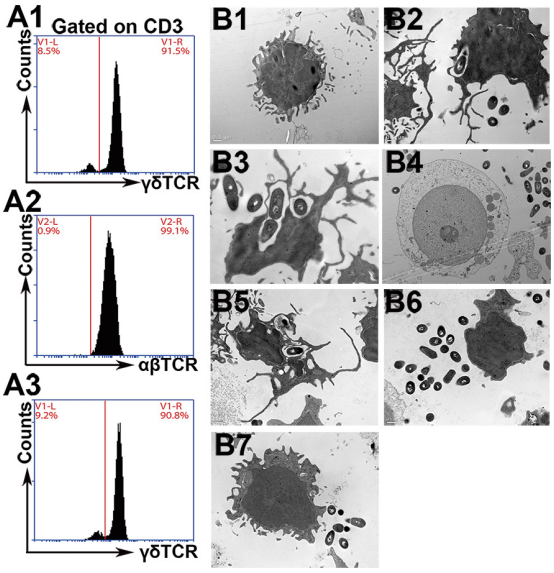

Figure 1. Human γδ T cells possess phagocytic capacity. (A) FCM analysis of γδ T cells and αβ T cells before incubation with *L. monocytogenes*. (A1) The percentage of γδ T cells in PBMCs at 10 d culture with RPMI 1640 medium containing IL-2 after stimulation in anti-pan-TCRγδ mAb-coated plates for five days. (A2) The percentage of αβ T cells after stimulation with CD3 and CD28 antibodies for four days. (A3) FCM analysis of naïve γδT cells sorted from fresh isolated PBMCs. (B) Representative TEM images of *L. monocytogenes* phagocytosis. (B1) Activated γδ T cells displayed "hairy" appearance after 10 d culture with anti-γδ TCR antibody and IL-2. (B2) Activated γδ T cells packaged *L. monocytogenes* bacterium by pseudopod-like plasma membrane after one hour of co-culture. (B3) Activated γδ T cells phagocytized *L. monocytogenes* three hours after incubation. (B4) Necrotic γδ T cells with *L. monocytogenes* in plasma membrane after co-culture for five hours. (B5) Rested γδ T cells display small but similar phagocytosis. (B6) Naïve γδ T cells did not phagocytize *L. monocytogenes*. (B7) Activated αβ T cells did not uptake *L. monocytogenes*. Scale bars = 0.5 μm.

Next, we determined whether naïve circulating γδ T cells could also phagocytose *L. monocytogenes*. Freshly isolated γδ T cells (purity > 90%, Figure 1A3) were incubated with *L. monocytogenes* in the same conditions as activated or rested γδ T cells. We observed no phagocytized *L. monocytogenes* in naïve γδ T cells up to three hours later (Figure 1B6). In addition, we found that αβ T cells, activated by CD3 and CD28 antibody (Figure 1A2), did not phagocytose *L. monocytogenes* either (Figure 1B7). These results suggest that human activated and rested γδ T cells, but not naïve γδ T cells, possess the ability to phagocytose pathogenic antigens, an important phenotype of APCs.

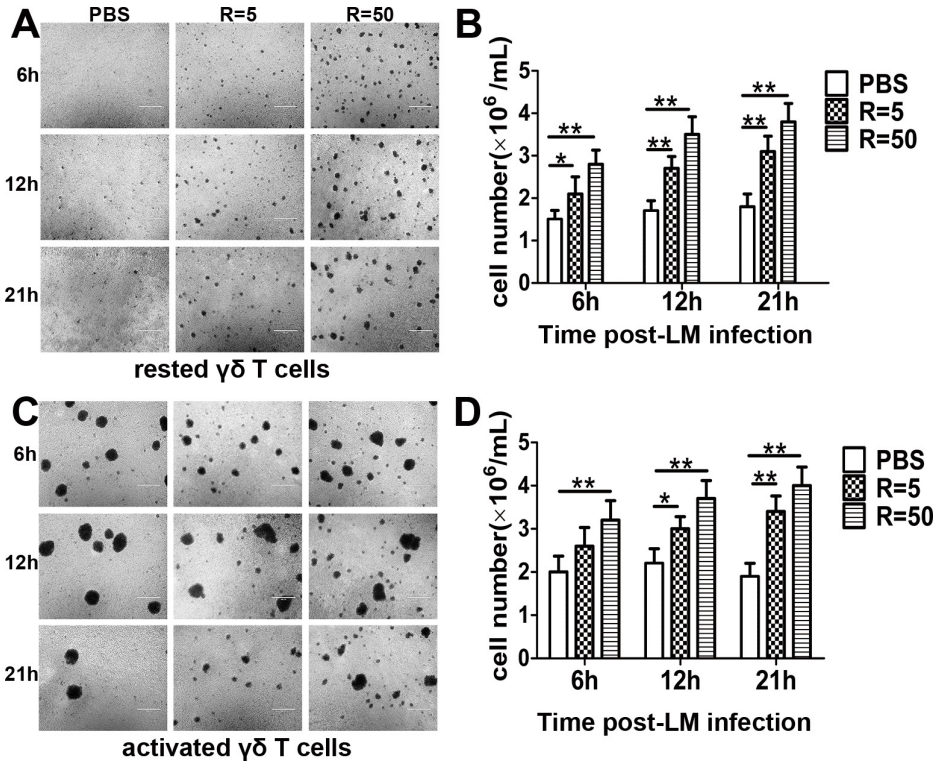

Figure 2. γδ T cells proliferated to form colonies after incubation with *L. monocytogenes*. (A) Many new and small colonies were observed after rested γδ T cells were incubated with *L. monocytogenes*. R is the ratio of *L. monocytogenes* to γδ T cells. PBS was used as control. More bacteria induced more colonies of γδ T cells. (B) Quantification of total cell numbers of rested γδ T cells in different groups. (C) Activated γδ T cells gathered to many large colonies 14 d after culture. After incubation with *L. monocytogenes*, some small and new colonies appeared. Over 21 h the number of large colonies decreased and small new colonies grew in size and number. Scale bars = 100 μm. (D) Quantification of total cell numbers of activated γδ T cells in different groups. Data are shown as mean ± SEM.*$P < 0.05$. **$P < 0.01$. (Independent experiments: n = 5).

L. monocytogenes Infection Induced Human γδ T Cell Proliferation

We observed a dramatic proliferation of γδ T cells after incubation with *L. monocytogenes* (Figure 2). Many small colonies were observed in rested γδ T cells 6 h after incubation (Figure 2A). The size and number of γδ T cells displayed in an *L. monocytogenes* dose-dependent manner. For example, at 12 h, γδ T cells in PBS control were $1.7 \pm 0.24 \times 10^6$/mL, then increased to $2.7 \pm 0.28 \times 10^6$/mL ($p = 0.009$) when stimulated by *L. monocytogenes* at R = 5 (R represents ratio of the number of *L. monocytogenes* bacteria to γδ T cells) and to 3.5 ± 0.42 ($p = 0.003$) at R = 50. More *L. monocytogenes* (R = 50) induced larger and more numerous colonies of γδ T cells (Figures 2B, D). Activated γδ T cells grew normally to yield many large colonies after 14 d in culture in the presence of IL-2. However, when incubated with *L. monocytogenes*, activated γδ T cells formed new small colonies (Figure 2C). The total cell number of γδ T cells was significantly higher when incubated with *L. monocytogenes* ($2.2 \pm 0.34 \times 10^6$/mL in PBS versus $3.0 \pm 0.28 \times 10^6$/mL at R = 5, $p = 0.035$; PBS versus $3.7 \pm 0.42 \times 10^6$/mL R = 50, $p = 0.009$, at 12 h) (Figures 2B, D). These data show that *L. monocytogenes* induced the proliferation of human activated or rested γδ T cells.

L. monocytogenes Infection Upregulated Expression of Antigen Presenting Related Molecules on γδ T Cells

The APC-like phenotype of γδ T cells indicates they possess the ability to process and present antigens. Therefore, we examined the expression levels of antigen presenting related molecules on γδ T cells in response to *L. monocytogenes* infection. The expression of HLA-DR molecules on rested γδ T cells was undetectable before the incubation with *L. monocytogenes*. We found the expression of HLA-DR molecules significantly increased in a dose dependent manner between six hours to 12 h after incubation with *L. monocytogenes*. The mean fluorescence intensities (MFI) of HLA-DR was 1.0 ± 0.20 in PBS control, and reached 1.27 ± 0.21 when infected with five-fold *L. monocytogenes* (R = 5 $p = 0.188$). When R = 50, MFI was 1.59 ± 0.48, but $p = 0.34$. (Figures 3A, B). HLA-DR expression returned to basal level after 15 h (Figures 3A, B). γδ T cells activated by IL-2 expressed high levels of HLA-DR molecules but no further increase after *L. monocytogenes* infection (Figures 3C, D).

CD80 and CD86 are costimulatory factors that transfer required secondary signals to active αβ T cells. In our experiments, under all conditions, we did not detect CD80 expression on γδ T cell surface from three hours to 21 h (Figures 3E, F). Similarly, although activation increased and rest reduced expression of CD86, we did not find changes after *L. monocytogenes* infection (Figures 3G, H).

CCR7 is an important lymph node (LN)-homing receptor for APC function (24). Therefore, we examined whether γδ T cells express CCR7 in response to *L. monocytogenes* infection. We found no detectable level of CCR7 in rested or activated γδ T cells in the absence of *L. monocytogenes*. However, CCR7 expression rapidly increased in activated γδ T cells when incubated with *L. monocytogenes* at six hours, MFI of CCR7 rose from 0.06 ± 0.05 (PBS control) to 0.69 ± 0.11 (R = 5) and further to 1.05 ± 0.13 (R = 50), the p values were 0.01

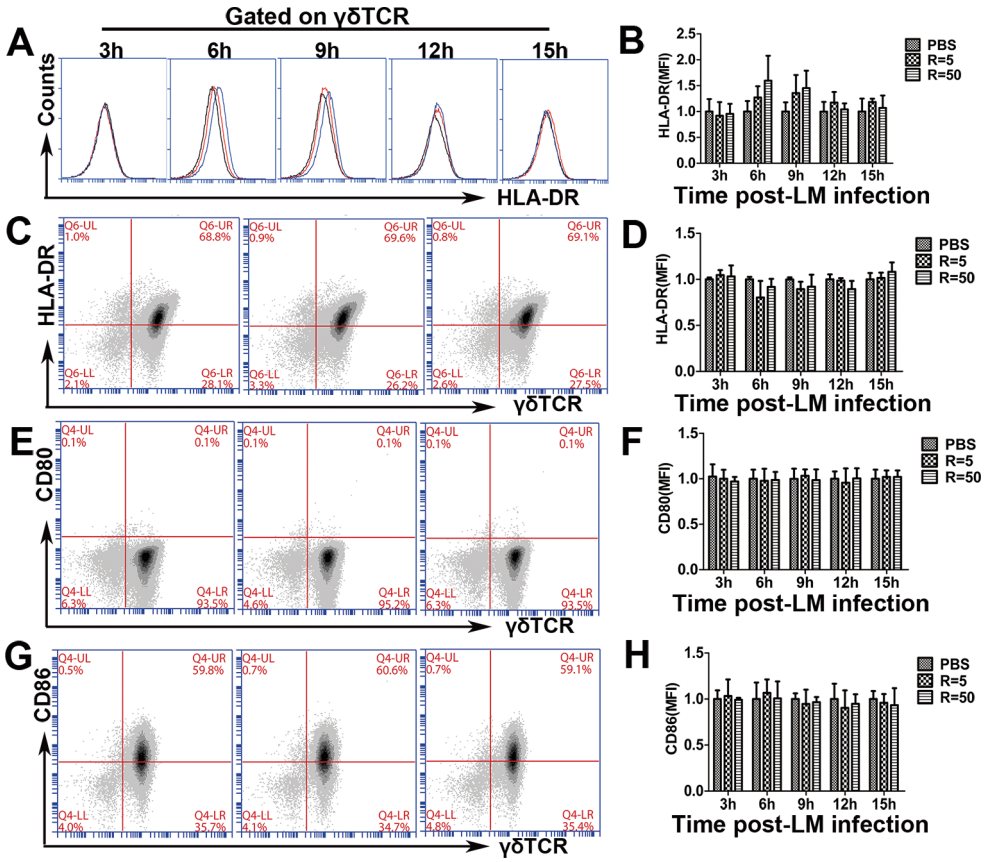

Figure 3. FCM analysis of antigen presentation related markers on γδ T cells in response to *L. monocytogenes* infection. (A) The level of HLA-DR expression increased on rested γδ T cells six hours after incubation with *L. monocytogenes*. (B) Quantification of normalized mean fluorescence intensities (MFI) of HLA-DR expression on rested γδ T cells in different groups. R represents the ratio of bacteria number to γδ T cell number. The high ratio of bacterium-to-cell of 50:1 (blue line) induced more HLA-DR expression on rested γδ T cells compared with low ratio (red line) or PBS (black line). No significant changes were observed at 12 h and15 h time points. (C, D, E, F, G and H) No significant change was observed in the expression level of HLA-DR (C and D), CD80 (E and F) or CD86 (G and H) on activated γδ T cells either in the presence or absence of *L. monocytogenes*. Data are shown as mean ± SEM (Independent experiments: n = 5).

and 0.000 respectively. (Figures 4A, B). This suggests activated γδ T cells have the potential to present antigens to effector cells with these antigen presenting molecules, costimulatory factors and LN-homing receptors.

Activated γδ T Cells Induced αβ T Cell Proliferation after *L. monocytogenes* Incubation

To determine whether γδ T cells act as APCs to induce primary αβ T cell responses, PBMCs were cocultured for six days with either *L. monocytogenes*, activated γδ T cells or *L. monocytogenes*-infected-γδ T cells at a ratio of γδ T cells to PBMCs of 1:1 or 1:10. The proliferation of PBMCs was examined by counting the cell number after six days. We found no obvious proliferation of T cells when PBMC were cultured alone (0.42 ± 0.07) or cocultured with *L. monocytogenes* (0.32 ± 0.08). However, the number of T cells significantly increased when PBMCs were cocultured with γδ T cells or *L. monocytogenes*-infected-γδ T cells at the ratio of γδ T cells to PBMCs of 1:1 (0.32 ± 0.08 LM + PBMC versus 0.87 ± 0.15 γδ T + PBMC, $p = 0.001$; LM + PBMC versus 1.16 ± 0.16 γδ T + LM + PBMC, $p = 0.000$; LM + PBMC versus γδ T + LM + PBMC, $p = 0.019$) and 1:10 (0.38 ± 0.14 LM + PBMC versus 0.76 ± 0.13 γδ T + PBMC, $p = 0.004$; LM + PBMC versus 0.99 ± 0.16 γδ T + LM + PBMC, $p = 0.000$; LM + PBMC versus γδ T + LM + PBMC, $p = 0.003$) (Figure 5A). We also analyzed the percentages of different subsets of T cells after six days using flow cytometry. The results show that the ratios (proliferated cells of a specific subset were divided by the initial cell number of PBMCs which eliminated the bias due to different initial cell numbers) of αβ T cells (Figure 5B), CD4 + T cells (Figure 5C) and CD8 + T cells (Figure 5D) significantly increased when cocultured with *L. monocytogenes*-infected-γδ T cells (1.22 ± 0.21 αβ T cells, 0.73 ± 0.17 CD4 + T cells, 0.48 ± 0.08 CD8 + T cells,

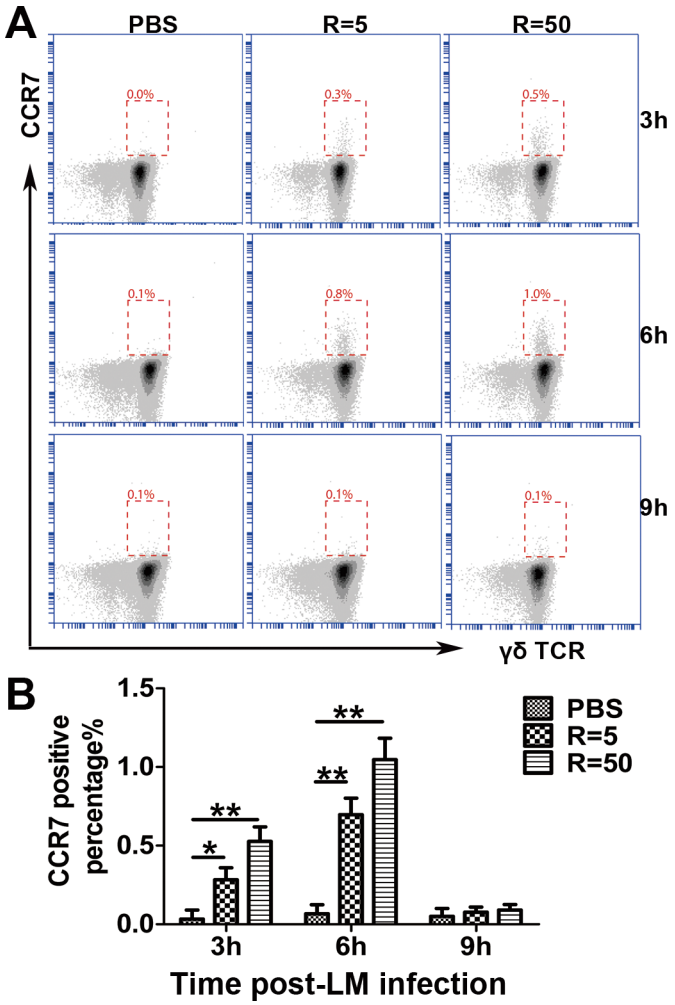

Figure 4. FCM analysis of CCR7 on activated γδ T cells after phagocytosed *L. monocytogenes*. The expression of CCR7 on activated γδ T cells was upregulated in a bacteria dose dependent manner three hours after *L. monocytogene* infection. The peak of CCR7 expression was at six hours and gradually decreased at nine hours. Normalized mean fluorescence intensity (MFI) values are shown as mean ± SEM (independent experiment of n = 5). **P* < 0.05.

for ratio = 1:1; 0.72 ± 0.07 αβ T cells, 0.43 ± 0.07 CD4 + T cells, 0.27 ± 0.03 CD8 + T cells, for ratio = 1:10) compared with PBMCs only or *L. monocytogenes*-infected-PBMCs (0.58 ± 0.07 αβ T cells, 0.27 ± 0.10 CD4 + T cells, 0.19 ± 0.04 CD8 + T cells, for ratio = 1:1; 0.36 ± 0.10 αβ T cells, 0.23 ± 0.05 CD4 + T cells, 0.17 ± 0.04 CD8 + T cells, for ratio = 1:10). Interestingly, in the absence of *L. monocytogenes*, γδ T cells alone also promoted the proliferation of αβ T cells (0.92 ± 0.15 for ratio = 1:1, 0.47 ± 0.09 for ratio = 1:10), $CD4^+$ T cells (0.48 ± 0.10 for ratio = 1:1, 0.32 ± 0.06 for ratio = 1:10), and $CD8^+$ T cells (0.33 ± 0.04 for ratio = 1:1, 0.18 ± 0.04 for ratio = 1:10) even though this effect was stronger when cocultured with *L. monocytogenes*-infected γδ T cells (γδ T + LM + PBMC versus LM + PBMC for αβ T cells, CD4 + T cells and CD8 + T cells, at ratio = 1:1, p = 0.004, p = 0.011, p = 0.003, respectively; at ratio = 1:10, p = 0.002, p = 0.05, p = 0.01, respectively). Without γδ T cells, PBMCs were cultured alone or with *L. monocytogenes*, only partial αβ T cells survived (Figures 5B–D). To verify this effect of γδ T cells, αβ T cells were purified from PBMCs and subjected to the same experiments. The results confirmed that γδ T cells alone promoted the proliferation of αβ T cells, especially in the presence of *L. monocytogenes* (0.23 ± 0.11 LM + PBMC versus 1.16 ± 0.13 γδ T + LM + PBMC, p = 0.004; LM + PBMC versus 0.81 ± 0.10 γδ T + PBMC, p = 0.009, at ratio = 1:1) (Figure 5E).

Activated γδ T Cells Induced αβ T Cell Differentiation after *L. monocytogenes* Incubation

The finding that γδ T cells promoted proliferation of αβ T cells after *L. monocytogenes* infection led us to investigate whether γδ T cells could induce the differentiation of naïve αβ T cells. We cocultured PBMCs with *L. monocytogenes*, γδ T cells or *L. monocytogenes*-infected γδ T cells and detected the differentiation of $CD4^+$ and $CD8^+$ αβ T cells after stimulation with Phorbol-12-myristate-13-acetate (PMA) and ionomycin (Ion). γδ T cells induced naïve $CD4^+$ and $CD8^+$ αβ T cells to polarize into effector cells, especially in the presence of *L. monocytogenes* (Figure 6). $CD4^+$ αβ T cells tended to produce IFN-γ (15.75 ± 3.32 γδ T + LM + PBMC versus 4.03 ± 1.16 LM + PBMC, p = 0.009; 13 ± 1.57 γδ T + PBMC versus LM + PBMC, p = 0.001; γδ T + LM + PBMC versus γδ T + PBMC, p = 0.28; at ratio = 1:1) (Figures 6A, C) rather than IL-4 or IL-17 (data not shown). This suggests *L. monocytogenes*-infected γδ T cells induce CD4 + T cells to T helper 1 (Th1)-type T cells rather than Th2 or Th17 cells. In addition, we found *L. monocytogenes*-infected γδ T cells induced $CD8^+$ αβ T cells to produce IFN-γ (9.73 ± 1.17 γδ T + LM + PBMC versus 4.7 ± 0.2 LM + PBMC, p = 0.002; 4.77 ± 1.15 γδ T + PBMC versus LM + PBMC, p = 0.93, at ratio = 1:1) (Figures 6B, D), indicating a direction of the differentiation to cytotoxic T lymphocytes (CTL). Interestingly, activated γδ T cells induced naïve $CD4^+$ αβ T cells but not naïve $CD8^+$ αβ T cells to produce IFN-γ. These results, taken together, suggest

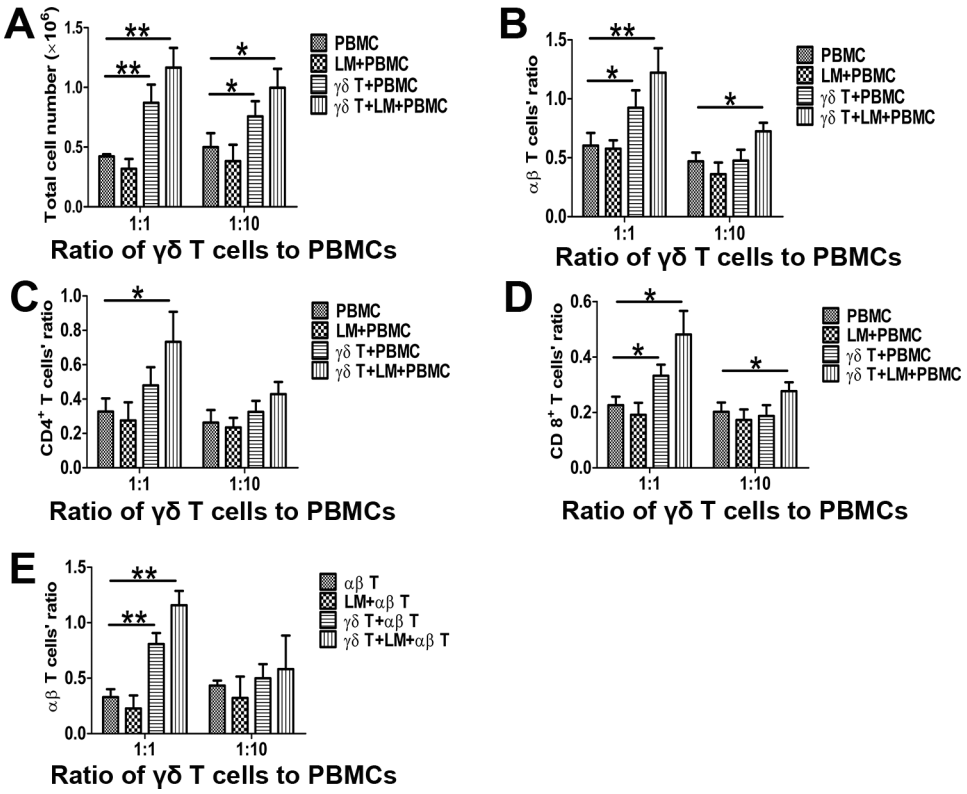

Figure 5. Phagocytized *L. monocytogenes*, activated γδ T cells to induce CD4 cell and CD8 T cell proliferation. (A) PBMC, PBMC plus *L. monocytogenes*, PBMC plus γδ T cells or PBMC plus γδ T cells infected by *L. monocytogenes* were cultured for six days then the total cell number in each group was counted. *L. monocytogenes* alone did not promote PBMC proliferation. γδ T cells displayed a slight augment to PBMC proliferation at a high ratio of γδ T cells to PBMCs. However, the γδ T cells which phagocytized *L. monocytogenes* induced significant PBMC proliferation. The ratios of the numbers of proliferated (B) αβ T cells, (C) $CD4^+$ T cells and (D) $CD8^+$ T cells to initial PBMC numbers after six days incubation. A more significant proliferation was observed when γδ T cells were cultured at 1:1 ratio of γδ T cells to PBMCs. (E) αβ T cells were isolated from PBMC and cocultured with γδ T cells in the presence or absence of *L. monocytogenes*. γδ T cells could promote the proliferation of αβ T cells, especially after phagocytosed *L. monocytogenes*. The cell number, ratio and percentage are shown as mean ± SEM (independent experiment of n = 4). $^*P < 0.05$. $^{**}P < 0.01$. Ratios represent as the proportions of the final cell numbers after incubation over initial numbers of PBMCs.

that $CD4^+$ αβ T cells were induced into Th1 cells and $CD8^+$ αβ T cells into CTLs in the presence of *L. monocytogenes*-infected-γδ T cells.

L. monocytogenes Infection Decreased the Percentage of γδ T Cells in Mouse IELs and Increased MHC-II Expression in γδ T Cells In Vivo

To determine whether *L. monocytogenes* activates γδ T cells *in vivo*, we characterized the phenotypes of γδ T cells in the IELs from the mice intragastrically infected with *L. monocytogenes*. The results show that the percentage of γδ T cells in the IELs decreased in the *L. monocytogenes*-infected mice compared with the controls ($P > 0.05$, Figures 7A, B). MHC-II expression significantly increased in γδ T cells from *L. monocytogenes*-infected mice compared with the controls (1.65 ± 0.35 PBS versus 6.0 ± 0.9 LM, $p = 0.046$, at 36 h after infection; 1.9 ± 0.1 PBS versus 7.6 ± 0.4 LM, $p = 0.005$, at 48 h after infection; Figures 7C, D). However, no obvious changes were found in the expression levels of other antigen presentation associated molecules including CD80, CD86 and CCR7 (data not shown). These data indicate that *L. monocytogenes* infection induces a mild activation of γδ T cells *in vivo* with a significant difference in the phenotype of γδ T cells in *L. monocytogenes* infection between human and mouse.

DISCUSSION

Clinical cases of listerelosis provide clues to the interaction of γδ T cells and *L. monocytogenes*. In Bridgett's report, *L. monocytogenes* bacterial infections induced multiple effector immune responses of activated γδ T cells in *L. monocytogenes*-infected macaques, including 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 (15). Recently, Romagnoli *et al.* reported IL-17A-producing resident

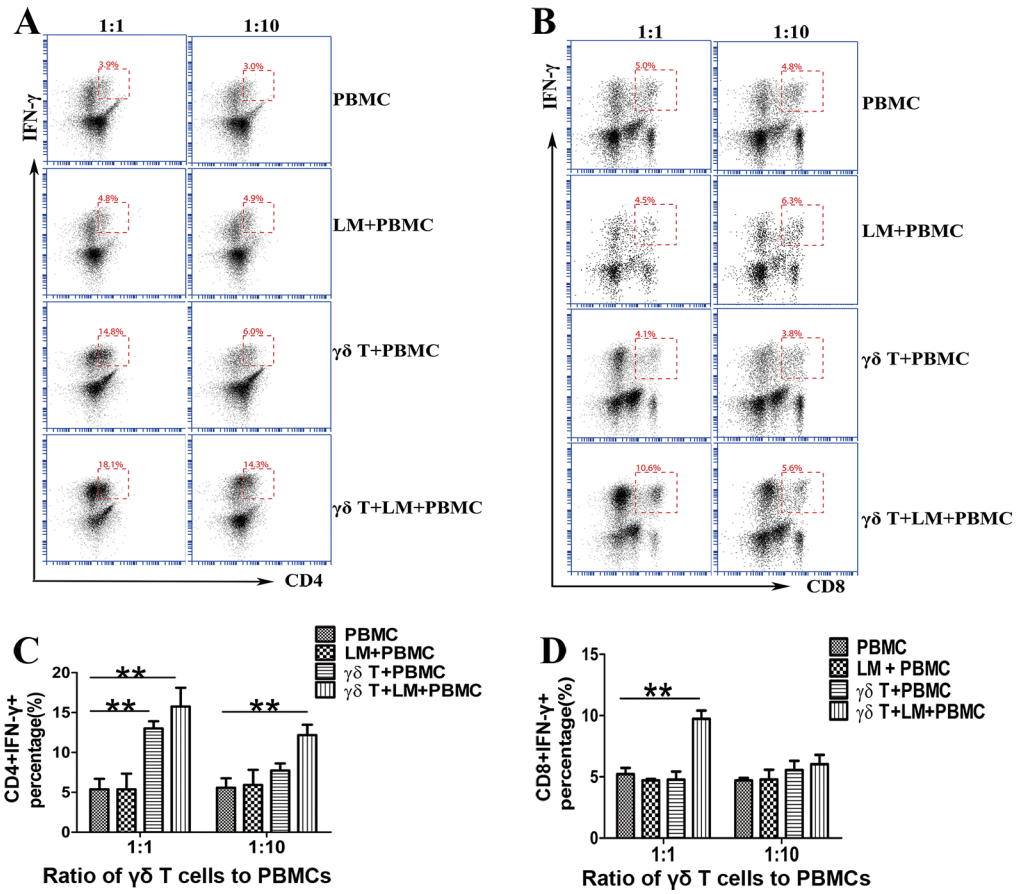

Figure 6. The differentiation of $CD4^+$ and $CD8^+$ T cells were induced by γδ T cells which phagocytosed *L. monocytogenes*. FCM analysis of intracellular IFN-γ expression in (A) $CD4^+$ T cells and (B) $CD8^+$ T cells after stimulation with PMA + Ion for two hours and blockage with BFA for four hours. After phagocytized *L. monocytogenes*, γδ T cells induced $CD4^+$ T cells and $CD8^+$ T cells to express IFN-γ at a high ratio of γδ T cells to responder cells. (C) Quantitation of the percentages of IFN-γ secreting $CD4^+$ T cells in different treatments. (D) Quantitation of the percentages of IFN-γ secreting $CD8^+$ T cells in different treatments. Data are shown as mean ± SEM from four independent experiments. $^*P < 0.05$. $^{**}P < 0.01$.

memory γδ T cells exhibited a remarkably static pattern of migration that radically changed following secondary oral L. monocytogenes infection (26).

In this study, we show a part of the activated and rested γδ T cells phagocytized *L. monocytogenes* bacteria. We hypothesized that it is due to different subpopulations of γδ T cells given no proliferation bias of subpopulations when activated by anti-γδ TCR antibody. Previous studies also reported that γδ T cells act as APCs including freshly isolated γδ T cells that phagocytized *E. coli* and 1 μm synthetic beads (10) and IPP-stimulated tonsillar γδ T cells that displayed principal characteristics of professional antigen presenting cells (9). Our findings show consistent results in activated γδ T cells and rested γδ T cells. However, we did not observe phagocytosis in the freshly isolated naïve γδ T cells. Professor Gustafsson regards CD16 as a γδ T cell phagocytic receptor (10). We know during the process of activation, γδ T cells lose CD16 expression (27,28) and upregulate the expression of MHC-II, CD80 and CD86 (9). All of these molecules are involved in antigen presentation; Gustafsson confirmed that activation increased phagocytosis and antigen presentation by γδ T cells. To further clarify these findings, we characterized phagocytized γδ T cells and the phagocytic receptor of activated γδ T cells.

We observed proliferation and colony forming in rested and activated γδ T cells after *L. monocytogenes* infection *in vitro*. In our experiments, live *L. monocytogenes* were added to γδ T cells to strongly activate γδ TCR and stimulate γδ T cell proliferation. After more than three hours, many γδ T cells died from necrosis, a phenomenon possibly caused by extracellular bacteria and/or their soluble products in cell culture medium or the uptake of *L. monocytogenes* (21,29). In addition, we found that phagocytosis triggered γδ T cells to rapidly,

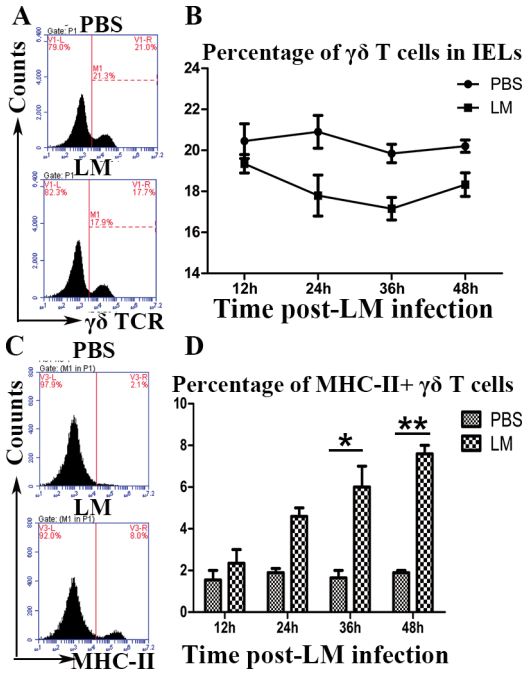

Figure 7. *L. monocytogenes* infection activated the expression of MHC-II molecules on γδ T cells. FCM analysis of percentage of γδ T cells in mouse IEL (A) and MHC-II + γδ T in γδ T cells (C) after intragastric administration with *L. monocytogenes* for 48 h. (B) Quantitation of the percentages of γδ T cells in mouse IELs. (D) Quantitation of the percentages of HLA-DR + γδ T cells in γδ T cells (gated in γδ T cells). Data are shown as mean ± SEM from four independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

but transiently, increase CCR7 expression, and sustained high expression of HLA-DR and costimulatory factor CD86. The expression of CCR7 enables γδ T cells to home lymph nodes and then engage in antigen presentation. Rested γδ T cells began to increase HLA-DR expression after *L. monocytogenes* infection for six hours, but did not express CCR7 and showed only low expression of CD86. Neither activated nor rested γδ T cells expressed CD80 as dendritic cells (DCs) did. In many cases, the expressions of CD80 and CD86 were inconsistent. Although both CD80 and CD86 are costimulatory signals, CD86 is more important (30). These results indicate that activated γδ T cells are more effective in APCs function.

Finally, we demonstrated that activated γδ T cells induced naïve $CD4^+$ or $CD8^+$ αβ T cells to proliferate and differentiate after *L. monocytogenes* phagocytosis. Both $CD4^+$ and $CD8^+$ αβ T cell numbers increased, and IFN-γ production was activated. CTLs lysed infected cells directly and Th1 cells induced apoptosis, which induced the battle of cleaning *L. monocytogenes*. The proliferation response of $CD8^+$ αβ T cells may be triggered by the antigen cross-presentation activity of γδ T cells as described previously (8,31).

We also note that at high incubation ratio, activated γδ T cells stimulated $CD4^+$ and $CD8^+$ αβ T cells to proliferate and differentiate. This phenomenon was also presented in Mao's paper, which showed peripheral-derived γδ T cells stimulated primary $CD4^+$ and $CD8^+$ T cells to proliferation on day three (23). Although γδ TCR and αβ TCR recognized different ligands and required different costimulated factors, they share partial common activation signal pathways (such as extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinases (MAPK) pathway), translation factor activation and IL-2 production (32), so the bystander effect only occurs at high ratios of γδ T cells with αβ T cells or an extended incubation period.

In this study we also characterized the phenotype changes of γδ T cells from mice infected by *L. monocytogenes*. However, we observed only a slight decrease in the percentage of γδ T cells in the IELs and a mild elevation of MHC-II expression on γδ T cells after *L. monocytogenes* infection. These findings suggest that γδ T cells are activated by *L. monocytogenes* infection and play a role in the process of antigen presentation (33). However, we found that *L. monocytogenes* infected mice showed no obvious changes in the expression levels of other antigen presentation associated molecules including CD80, CD86 and CCR7. This indicates that there is a significant difference in the phenotype changes of γδ T cells in *L. monocytogenes* infection between human and mouse.

In summary, we show activated and rested γδ T cells are able to phagocytize *L. monocytogenes*. This phagocytosis leads to antigen processing and presentation. This is a helpful supplement to understanding the multiple effect functions of activated γδ T cells in *L. monocytogenes* infection. Furthermore, these findings suggest that γδ T cells may be potential targets for immunotherapy. Our hope is that more researchers will focus on the antigen presenting function of γδ T cells in anti-infection or antitumor immunity and translate discoveries into effective therapeutic approaches in cancer patients.

CONCLUSION

Overall, our study highlights the mechanism of human γδ T cells to serve as APCs during the infection of *L. monocytogenes*, which are common foodborne bacterial pathogens. The bacteria produce metabolite products recognized by γδ TCRs and results in γδ T cell overrepresentation during *L. monocytogenes* infection. In this study, we observed via transmission electronic microcopy that γδ T cells phagocytize *L. monocytogenes*. Upon stimulation with *L. monocytogenes*, γδ T cells increased

surface expression of activation markers (HLA-DR and CCR 7) present antigens and induce the proliferation and differentiation of homologous αβ T cells. *In vivo* experiments showed that *L. monocytogenes* infection activated the expression of MHC-II molecules in γδ T cells. These findings indicate that human γδ T cells display APC functions during *L. monocytogenes* infection. These finding are beneficial to the develop γδ T cell therapeutic applications in bacterial infection or tumor development.

ACKNOWLEDGMENTS

This study was supported by the National Program for Key Basic Research Projects (2013CB530503), National Natural Science Foundation of China (81471574, 91542117, 81673010), the Mega-Projects of National Science Research for the 12th Five-Year Plan (2012ZX10001006), the Ministry of Health (201302018 and 201302017) and the National Key Research and Development Program of China (2016YFA0101001). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. We thank Dr. Austin Cape at ASJ Editors for careful reading and comments.

DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Cite this article as: Zhu Y, *et al.* (2016) Human γδ T cells augment antigen presentation in *listeria monocytogenes* infection. *Mol. Med.* 22:737–46.