Potential Role of Gr-1⁺ CD8⁺ T Lymphocytes as a Source of Interferon- γ and M1/M2 Polarization during the Acute Phase of Murine *Legionella pneumophila* Pneumonia

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Abstract

In this study, we analyzed interferon (IFN)- γ -producing cells and M1/M2 macrophage polarization in *Legionella pneumophila* pneumonia following anti-Gr-1 antibody treatment. Anti-Gr-1 treatment induced an M1-to-M2 shift of macrophage subtypes in the lungs and weakly in the peripheral blood, which was associated with increased mortality in legionella-infected mice. CD8⁺ T lymphocytes and natural killer cells were the dominant sources of IFN- γ in the acute phase, and anti-Gr-1 treatment reduced the number of IFN- γ -producing CD8⁺ T lymphocytes. In the CD3-gated population, most Gr-1-positive cells were CD8⁺ T lymphocytes in the lungs and lymph nodes (LNs) of infected mice. Additionally, the number of IFN- γ -producing Gr-1⁺ CD8⁺ T lymphocytes in the lungs and LNs increased 2 and 4 days after *L. pneumophila* infection, with anti-Gr-1 treatment attenuating these populations. Antibody staining revealed that Gr-1⁺ CD8⁺ T lymphocytes were Ly6C-positive cells rather than

Ly6G, a phenotype regarded as memory type cells. Furthermore, the adoptive transfer of Gr-1⁺ CD8⁺ T lymphocytes induced increases in IFN- γ , M1 shifting and reduced bacterial number in the *Legionella* pneumonia model. These data identified Ly6C⁺ CD8⁺ T lymphocytes as a source of IFN- γ in innate immunity and partially associated with reduced IFN- γ production, M2 polarization, and high mortality in anti-Gr-1 antibody-treated mice with *L. pneumophila* pneumonia.

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Introduction

Legionella spp. is a Gram-negative intracellular pathogen that causes severe pneumonia [1, 2]. This organism coexists with freshwater amoebae and infects humans through the inhalation of contaminated water and aerosols [3–5]. The majority of *Legionella* infections is caused by *L. pneumophila* serogroup-1 [6, 7], with the most important pathogenic property of this organism believed to be resistant to intracellular killing and proliferation within phagocytic cells, especially macrophages and monocytes [8–10]. *L. pneumophila* possess the Dot/Icm type IV

secretion system, which translocates effector proteins into host cells [11]. These effectors not only play essential roles in the biogenesis of the specialized replicative organelle but also function as messengers that trigger and modulate host immune responses [12].

Macrophages are an essential component of innate immunity and play a central role in inflammation and host defense against a variety of organisms, including intracellular pathogens [13]. Several subpopulations of macrophages exist [14]. In response to various signals, macrophages differentiate into classical M1 activation (in response to Toll-like receptor ligands and interferon [IFN]- γ) or alternative M2 activation (stimulated by interleukin [IL]-4/IL-13) [15, 16]. The M1 phenotype is characterized by the expression of high levels of proinflammatory cytokines, elevated production of reactive nitrogen and oxygen intermediates, promotion of T helper (Th)1 response, and strong microbicidal and tumoricidal activity. By contrast, M2 macrophages are involved in immunoregulatory functions, such as parasite containment, tissue remodeling, and tumor progression. Although Th1-type host responses and cytokine production, such as increases in IFN- γ and IL-12, were demonstrated in the serum of *Legionella* pneumonia patients [17], the consequences of macrophage M1/M2 polarization on disease outcome remains unclear.

Previously, we demonstrated that early recruitment of neutrophils, probably through a cellular source of IL-12, contributed to Th1 polarization in a murine model of *L. pneumophila* pneumonia [18]. Anti-Gr-1 antibody treatment induced immunological shift from the Th1 to Th2 lymphocyte subset, which was characterized by decreased IFN- γ /IL-12 and increased IL-4/IL-10 [18]. Subsequently, the expression of Gr-1 antigen has been reported in several immune cells, including dendritic cells (DCs) and CD8⁺ T cells, in addition to neutrophils [19, 20]. The precise mechanisms of reduced IFN- γ production and increased susceptibility to lethal infection following anti-Gr-1 antibody administration might involve multiple cells and factors in the network of host antibacterial-defense systems.

In this study, we examined the effects of the anti-Gr-1 antibody on M1/M2 macrophage polarization. Our data demonstrated that *L. pneumophila* infection induced M1 macrophage skewing, whereas anti-Gr-1 antibody treatment reduced IFN- γ production, promoted M2 phenotype macrophage differentiation, and increased legionella-induced lethality. These changes were associated with reductions in the number of Gr-1⁺ CD8⁺ T lymphocytes, specifically Ly6C⁺ CD8⁺ T lymphocytes, which were shown to

be a major source of IFN- γ in CD3-gated cells during the acute phase of infection. Although Ly6C expression represents as a marker of memory CD8 T cells, we observed that it also played an important role in the acute phase of infection. These data suggested that IFN- γ -producing Gr-1⁺ CD8⁺ T lymphocytes might be key cellular players in governing M1/M2 polarization and determining outcomes in acute lethal *L. pneumophila* pneumonia.

Materials and Methods

Animals

Female, specific pathogen-free 6- to 10-week-old A/J mice were purchased from Sankyo Laboratory (Tokyo, Japan). All mice were housed in specific pathogen-free conditions within the animal care facility at Toho University School of Medicine (Tokyo, Japan) until the day of sacrifice. Animal and pathogen protocols were approved by the institutional care and use committee (approval number 17-55-220).

L. pneumophila Inoculation and Determination of Bacterial Number

Clinical isolates of *L. pneumophila* Suzuki, a serogroup-1 strain stocked at Toho University Hospital [2, 17], were prepared as previously reported [21]. Animals were anesthetized intramuscularly with ketamine at 50 mg/kg body weight and xylazine at 10 mg/kg. Their tracheas were exposed, and 30 μ L of bacterial suspension was administered via a sterile 26-gauge needle. Skin incisions were closed with surgical staples. For determination of bacterial number at the indicated time points, lungs were homogenized with a homogenizer (IKA Japan K.K., Osaka, Japan) in 1 mL of saline. Portions of homogenates (10 μ L) were inoculated onto buffered charcoal yeast extract agar supplemented with α -ketoglutaric acid after serial 1:10 dilutions in saline. Agar plates were incubated at 35 °C for 3–4 days, after which colonies were counted visually.

Preparation of Bone Marrow-derived Macrophages (BMDMs)

BMDMs were isolated from the femurs of female A/J mice, as previously described [22]. Marrow cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, penicillin-streptomycin, 2-mercaptethanol, L-cell-conditioned medium, and 2 ng/mL recombinant murine macrophage colony stimulating factor (R&D Systems, Minneapolis, MN, USA) for 4 days, as previously described [23].

In vitro Cytokine Stimulation and Stimulation of BMDMs with *L. pneumophila* and Determination of Bacterial Number

On day 4, the BMDM culture medium was replaced with fresh medium, resulting in a final concentration of 20 ng/mL IFN- γ or 150 ng/mL IL-4, and cells were cultured for an additional 24 h. Cultures were split, replated at 1×10^6 cells per well in a 6-well plate for flow cytometry or 1×10^5 cells per well in a 96-well plate for determination of bacterial number. *L. pneumophila* cells were cultured, rinsed, and enumerated as described. Macrophages were infected at a multiplicity of infection of 0.1, attached by centrifuga-

tion, and incubated for 2 h, followed by washing away extracellular bacteria. After incubation for 24 h, cells were collected for flow cytometry. After incubation for 24 or 48 h, culture supernatants were collected, and the infected macrophages were lysed to determine the bacterial number, as previously described [24].

Isolation of Lung Cells and Flow Cytometric Analysis

The excised lung tissue separated from all associated lymph nodes (LNs), was minced and incubated at 37 °C in 5% CO₂ for 50 min in Roswell Park Memorial Institute 1640 medium containing 2% fetal bovine serum, 0.5 mg/mL collagenase D (Roche, Basel, Switzerland) and 150 µg/mL DNase (Roche). Single-cell suspensions were prepared by passing them through a 70-µm cell strainer (Falcon; Thermo Fisher Scientific, Waltham, MA, USA). Cell suspensions with stain buffer (phosphate-buffered saline) containing 2% bovine serum albumin and 2 mM EDTA were incubated with an Fc-receptor-blocking antibody (anti-mouse CD16/32, clone 93) for 15 min on ice to reduce nonspecific antibody binding. Cells were then washed with stain buffer and surface stained for 30 min on ice using each experimental design combination of peridinin chlorophyll protein complex (PerCP)/Cy5.5 anti-mouse CD11b (clone M1/70), fluorescein isothiocyanate (FITC) anti-mouse Ly6G (clone 1A8), phycoerythrin (PE)/Cy7 anti-mouse F4/80 (clone BM8), PE anti-mouse CD86 (clone GL-1), allophycocyanin (APC) anti-mouse CD206 (clone C068C2), FITC anti-mouse CD3e (clone 145-2C11), APC/Cy7 anti-mouse CD3e (clone 145-2C11), PerCP anti-mouse CD4 (clone RM4-5), PE anti-mouse CD8a (clone 53-6.7), APC/Cy7 anti-mouse CD49b (clone DX5), APC/Cy7 anti-mouse CD44 (clone IM7), and FITC anti-mouse Gr-1 (clone RB6-8C5). Cells were washed, fixed with 4% paraformaldehyde, and detected using a FACSCantoII flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Intracellular Cytokine Staining

Intracytoplasmic cytokine staining was performed using the Cytofix/Cytoperm Plus kit according to the manufacturer's protocol (BD Biosciences). To assess intracellular cytokine expression, phosphomolybdic acid (PMA; 25 ng/mL), ionomycin (1 µg/mL), and GolgiPlug (1 µg/mL; BD Biosciences) were added to cells and incubated for 4 h. Cells were stained for cell-surface markers and fixed for 20 min on ice. After washing, cells were stained for intracytoplasmic IFN-γ expression with APC anti-mouse IFN-γ (clone XMG 1.2) or APC IgG isotype control diluted in Perm/Wash solution (BD Biosciences) for 30 min and detected using a FACSCantoII flow cytometer (BD Biosciences).

Gr-1⁺ Cell Depletion In vivo

For Gr-1⁺ cell depletion, we used the RB6-8C5 monoclonal antibody (mAb), a rat anti-mouse IgG2b, directed against Ly-6G and Ly6C (previously known as Gr-1), with isotype mAb was used as a control. A total of 100 µg of RB6-8C5 or control IgG was administered intraperitoneally 1 day prior to bacterial administration.

RNA Isolation and Gene-expression Analysis

Total RNA was isolated from lungs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. For quantitative polymerase chain reaction analysis,

1 µg of total RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Data analysis utilizing the SYBR Green real-time reverse transcription PCR technique was performed on an ABI Prism 7000 sequence detector system (Applied Biosystems). We used the following PCR primers: inducible nitric oxide synthase (iNOS), 5'-CCCTCCGAAGTTTCTGGCAGCAGC-3' (forward) and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (reverse); arginase-1(Arg-1)5'-CAGAAGAATGGAAGAGTCAG-3' (forward) and 5'-CAGATATGCAGGGAGTCACC-3' (reverse); IFN-γ, 5'-GAACTGGCAAAGGATGGTGA-3' (forward) and 5'-TGTGGGTGTTGACCTCAAAC-3' (reverse); IL-10, 5'-TTTGAATTCCCTGGGTGAGAA-3' (forward) and 5'-GCTCCACTGCCTTGCTCTTATT-3' (reverse); β-actin, 5'-AGAGGGAAATCGTGCCTGAC-3' (forward) and 5'-CAATAGTGATGACCTGGCCGT-3' (reverse). Relative fold changes in transcript levels were calculated using the 2^{-ΔΔCT} (where CT is threshold cycle) method [25] and using the housekeeping gene β-actin as a reference standard for the amount loaded and the quality of the cDNA.

Adoptive Transfer of Ly6C⁺ CD8⁺ T Lymphocytes into Anti-Gr-1 Antibody-Treated Mice with L. pneumophila Infection

We euthanized donor A/J mice and harvested multiple tissues, and processed axillary and mediastinal LNs and spleen into cell suspensions. First, we minced the tissues and passed the cells through 70-µm cell strainers (Falcon; Thermo Fisher Scientific). CD8 T cells were isolated using a pan T-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by a CD8a T-cell isolation kit (Miltenyi Biotec). Ly6C⁺ CD8T lymphocytes were then isolated using biotin-labeled anti-mouse Ly-6C antibody (Biolegend, San Diego, CA, USA) and streptavidin microbeads (Miltenyi Biotec). The cells were resuspended in phosphate-buffered saline, and approximately 1.5 × 10⁶ cells were transfused into the tracheae of anti-Gr-1 antibody-treated mice on the same day as *L. pneumophila* infection.

Statistical Analysis

All results are expressed as means ± SD. Student *t* test was used for statistical evaluations of normally distributed data using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at a 2-tailed value of *p* < 0.05.

Results

Effect of Anti-Gr-1 Antibody Treatment on Macrophage Subtypes in L. pneumophila Infection

Type I macrophages (M1) are proinflammatory, whereas type II macrophages (M2) are anti-inflammatory or pro-reparative [26]. We first examined the effects of anti-Gr-1 antibody administration on macrophage polarization in mice with *L. pneumophila* pneumonia. The lung cells from mice infected with *L. pneumophila* were harvested and analyzed by flow cytometry on days 2 and 3 after infection. We confirmed

Fig. 1. Effects of the anti-Gr-1 antibody on M1/M2 macrophage polarization in the lungs of mice with *Legionella pneumophila* pneumonia. **a** Anti-Gr-1 antibody or isotype antibody was administered intraperitoneally 1 day before infection, and then mice were intratracheally challenged with 1.2×10^5 CFU of *L. pneumophila*. Total lung cells were isolated from the mice on day 2 of infection and stained with antibodies to detect neutrophils (CD11b⁺, Ly6G⁺). **b** Total lung cells were isolated from mice on day 2 and under the same conditions as (a) and stained by antibodies to detect macrophages (CD11b⁺, F4/80⁺). Expression levels of CD86 (M1) and CD206 (M2) on macrophages were analyzed. **c** Proportions and cell number of CD86⁺ cells at 2 and 3 days after infection were calculated from data in (b). **d** Proportions and cell number of CD206⁺ cells at 2 and 3 days after infection were calculated from data in (b). Bars indicate the mean $\pm$ SD ($n = 4-5$ /group). Results were confirmed by 2 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

that almost all neutrophils accumulating in the lung were deleted by anti-Gr-1 administration (Fig. 1a) accompanied by a significant reduction in survival rate (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000490585), which agreed with results reported previously [18]. Subsequently, we analyzed macrophage subtype by flow cytometry using CD86 (M1) and CD206 (M2) as indices [27]. In macrophages (CD11b⁺, F4/80⁺), surface CD86 expression significantly increased on days 2 and 3 after infection, whereas CD86 expression decreased on day 3 after infection in anti-Gr-1-treated mice (Fig. 1b, c). Conversely, CD206 expression decreased during infection, with this loss blunted by anti-Gr-1 administration (Fig. 1b, d). Additionally, serum CD206 expression in the blood

significantly increased on day 3 after infection in anti-Gr-1-treated mice (online suppl. Fig. S2a, b). These results indicated that the shift from M1 to M2 occurs in the mouse lung simultaneous to survival exacerbation following *L. pneumophila* infection due to administration of the Gr-1 antibody.

Effects of Anti-Gr-1 Antibody on M1/M2-related Cytokine Productivity and Markers in *L. pneumophila* Infection

Next, we examined the gene expression of M1/M2 markers using iNOS for M1 and Arg-1 for M2 by real-time quantitative polymerase chain reaction (Fig. 2a) [26]. Consistent with flow cytometry results, anti-Gr-1 administration induced M2-like characteristics, includ-

Fig. 2. Effects of the anti-Gr-1 antibody on M1/M2-related cytokine production and markers in *Legionella pneumophila* infection. **a** mRNA levels of *iNOS* (M1) or *Arg-1* (M2) in the lungs were examined at 2- and 3-days post-infection. **b** mRNA levels of *IFN-γ* (M1) or *IL-10* (M2) in the lungs were examined at 2- and 3-days post-infection. Data are expressed as fold increase. All bars indicate the mean $\pm$ SD ($n = 3\text{--}4/\text{group}$). Results were confirmed by 2 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

ing a decrease in *iNOS* and increase in *Arg-1* mRNA expression. The lung cells with *Legionella* infection demonstrated strong M1 responses, characterized by significant increases in *IFN-γ* levels, whereas anti-Gr-1-treated mice exhibited significantly lower levels of *IFN-γ* and simultaneous elevations in levels of the M2 cytokine *IL-10* (Fig. 2b). As reported previously, anti-Gr-1 induces an immunological shift from Th1 to Th2 lymphocyte subsets characterized by Th1 (*IFN-γ*)/Th2 (*IL-10*) cytokine valances [18]. In the present study, our data showed that anti-Gr-1 administration induced a shift in macrophage subtypes from M1 to M2, as well as skewing from a Th1 to Th2 cytokine profile.

Effects of Exogenous *IFN-γ* or *IL-4* on M1/M2 Polarization and Anti-*L. pneumophila* Activity in BMDMs

We examined the effects of exogenous *IFN-γ* or *IL-4* on M1/M2 polarization and anti-*L. pneumophila* activity in BMDMs. During *L. pneumophila* infection, although macrophages after *IFN-γ* stimulation reportedly enhance bactericidal activity [24], the macrophage subtype present at that time was not reported. Here, we stimulated BMDMs with *IFN-γ* or *IL-4* for 24 h, followed by cell collection for flow cytometric analysis. Exogenous stimulation with *IFN-γ* or *IL-4* induced M1 or M2 polarization respectively (online suppl. Fig. S3a). We then measured changes in bacterial number in BM-

DMs induced in each subtype after 24 and 48 h of *L. pneumophila* infection (online suppl. Fig. S3b). M1 macrophages enhanced bactericidal activity at 48 h of incubation, whereas no significant changes in bacterial numbers were demonstrated in M2 macrophages as compared to the corresponding controls. These data suggested that *IFN-γ*-induced M1 macrophages enhanced bactericidal activity associated with *L. pneumophila* infection.

Reduction of CD8 T Cells in the Acute Phase of *L. pneumophila* Infection by Administration of the Gr-1 Antibody

To determine the mechanism associated with macrophage polarization following *L. pneumophila* infection by administration of the Gr-1 antibody, we analyzed cell population other than neutrophils, including lymphocytes, in the lung after antibody administration. Total cells were collected from the lungs of mice 2 days after infection, and flow cytometric analysis of lymphocytes showed that anti-Gr-1 administration did not affect CD4 T cells and natural killer (NK) cells, although the proportion of CD8 T cells decreased (Fig. 3a, b). These results suggested that CD8 T cells expressing the Gr-1 antigen on the cell surface were present in the lungs during the acute phase of infection and were excluded by administration of the Gr-1 antibody, similar to neutrophils.

Fig. 3. Reduction of CD8 T cells during the acute phase of *Legionella pneumophila* infection by administration of the Gr-1 antibody. **a** Total lung cells were isolated from mice on day 2 of infection and stained with antibodies to detect CD4 T cells (CD3⁺, CD4⁺), CD8 T cells (CD3⁺, CD8⁺), and NK cells (CD49b⁺). The proportion of each cell population was analyzed by gating according to lymphocyte size and complexity. **b** Proportions and cell number of CD8 T cells were calculated at 1- and 2-days post-infection. Bars indicate the mean $\pm$ SD ($n = 3$ /group). Results were confirmed by 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increase in Gr-1⁺ CD8⁺ T Lymphocytes in Mice during the Acute Phase of *L. pneumophila* Pneumonia

Gr-1 is a functionally important molecule expressed on granulocytes [28]. Additionally, previous studies showed that Gr-1 antigen is also expressed on CD8⁺ memory-type T lymphocytes as well as granulocytes, and that most of these cells are completely eliminated by the administration of the anti-Gr-1 antibody in vivo [20]. On the basis of these findings, we hypothesized that Gr-1 might be expressed on CD8⁺ T lymphocytes in the lungs of mice during the acute phase of *L. pneumophila* infection. As expected, in CD3-gated cells, Gr-1 antigen was expressed mainly on CD8⁺ T lymphocytes in the lungs and LNs of mice with *L. pneumophila* pneumonia, and Gr-1⁺ CD8⁺ T lymphocytes disappeared almost completely in anti-Gr-1-treated mice with infection (Fig. 4a, c). Moreover, the number of Gr-1⁺ CD8⁺ T lymphocytes in the lungs and LNs was significantly elevated

after *L. pneumophila* infection, with this number significantly reduced following Gr-1 antibody treatment (Fig. 4b, d).

Gr-1⁺ CD8⁺ T Lymphocytes as a Source of IFN- γ in the Acute Phase of *L. pneumophila* Pneumonia

We hypothesized that Gr-1⁺ CD8⁺ T lymphocytes are the source of IFN- γ -induced differentiation of macrophages into the M1 subtype during the acute phase of infection. To test this hypothesis, cells from infected mice were collected, and intracellular staining was performed. Cellular sources of IFN- γ include NK cells during the innate phase and Th1 cells/CD8⁺ T cells during the adaptive immune response [29]. However, although the main source of IFN- γ was indeed NK cells, CD8⁺ T cells also produced IFN- γ , despite the acute phase of infection. Interestingly, we found that CD8⁺ T cells expressing Gr-1 antigen mainly produced IFN- γ in both

Fig. 4. Increases of Gr-1⁺CD8 T lymphocytes in mice during the acute phase of *Legionella pneumophila* pneumonia. **a** Total lung cells were isolated from mice on days 2 and 4 of infection and stained with antibodies to detect Gr-1⁺CD8 T cells (CD3⁺, CD8⁺, Gr-1⁺). The proportion of the CD3⁺-gated population was analyzed by gating according to lymphocyte size and complexity. **b**

Proportions and cell number of Gr-1⁺CD8⁺ T cells were calculated from data in **(a)**. **c** LN cells were isolated from mice on days 2 and 4 and analyzed as described for lung cells. **d** Proportions and cell number of Gr-1⁺CD8⁺ T cells were calculated from data in **(c)**. All bars indicate the mean $\pm$ SD ($n = 4$ /group). Results were confirmed by 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

lungs (Fig. 5a, b) and LNs (Fig. 5c, d) after 2 or 4 days of infection. Additionally, anti-Gr-1 treatment clearly reduced the number of these cells in both the lungs and LNs. These data demonstrated that Gr-1⁺ CD8T⁺ lym-

phocytes were the source of IFN- γ during the acute phase of *L. pneumophila* infection and contributed to the change in macrophage subtype following administration of the Gr-1 antibody.

Fig. 5. Effects of Gr-1 antibody administration on IFN- γ production by Gr-1⁺CD8⁺ T lymphocytes in the lungs and LNs. **a** Total lung cells were isolated from mice on days 2 and 4 of infection, stimulated with PMA/ionomycin and GolgiPlug for 4 h, and stained with antibodies to detect Gr-1⁺CD8⁺ T cells (CD3⁺, CD8⁺, and Gr-1⁺), followed by intracellular staining for IFN- γ . The proportion of the CD3⁺-gated population was analyzed according to

lymphocyte size and complexity. **b** Proportions and cell number of Gr-1⁺CD8⁺ T cells were calculated from data in (a). **c** LN cells were isolated from mice on days 2 and 4 of infection and analyzed as described for lung cells. **d** Proportions and cell number of Gr-1⁺CD8⁺ T cells were calculated from data in (c). All bars indicate the mean $\pm$ SD ($n = 4$ /group). Results were confirmed by 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effects of Adoptive Transfer of Gr-1⁺ CD8⁺ T Lymphocytes on Macrophage Polarization and Bacterial Number in the Lungs

The anti-Gr-1 antibody recognizes the common epitope of Ly6G and Ly6C molecules [28]. Furthermore,

CD8 T cells expressing Ly6C are memory type [20]. Therefore, we investigated whether CD8 T lymphocytes expressing Gr-1 antigen harbor the Ly6C epitope. Cells were isolated from mouse lungs on day 2 of *L. pneumophila* infection and stained for surface markers of

Fig. 6. Effects of adoptive transfer of Gr-1⁺CD8T⁺ lymphocytes on macrophage polarization and bacterial number in the lungs. **a** Total lung cells were isolated from mice on day 2 of infection and stained with anti-Ly6G, anti-Ly6C, and anti-CD44 to examine the surface antigen of Gr-1⁺CD8⁺ T cells. **b** Ly6C⁺CD8⁺ T cells were purified from the spleen and LNs of naïve mice using a magnetic column. The cells were resuspended in PBS, $\sim 1.5 \times 10^6$ cells were transfused into the trachea on the same day as *Legionella pneumophila* infection in anti-Gr-1-treated mice. Three days after adoptive transfer, IFN- γ mRNA levels in the lung were examined. **c** mRNA levels of CD86 (M1 marker) and CD206 (M2 marker) in the lungs were examined 3 days after adoptive transfer. **d** Number of bacteria in the lungs was enumerated 3 days after adoptive transfer. Results were confirmed by 2 independent experiments. * $p < 0.05$; *** $p < 0.001$.

IFN- γ -producing cells. We observed that these cells expressed the Ly6C but not the Ly6G, epitope (Fig. 6a). To investigate the role of Ly6C⁺ CD8 T cells in vivo, these cells were purified from the spleens and LNs of naïve mice using a magnetic column and adoptively transferred intratracheally on the same day as *L. pneumophila* infection of mice administered Gr-1 antibody. Three days after adoptive transfer, IFN- γ productivity in lung tissue was predominantly elevated in the adoptive-transfer group as compared with the control group (no adoptive transfer; Fig. 6b). Evaluation of macrophage subtype revealed increases in CD86 expression (an M1 marker, $p = 0.27$) and decreases in CD206 expression (an M2 marker, $p = 0.22$) in the adoptive-transfer group relative to controls. Although there was no significant difference, the result indicated a tendency for macrophages to shift to M1 (Fig. 6c). Additionally, the adoptive-transfer group exhibited a significant decrease in the number of lung bacteria (Fig. 6d). These results demonstrated that Ly6C⁺ CD8 T cells were the source of

mophila infection in anti-Gr-1-treated mice. Three days after adoptive transfer, IFN- γ mRNA levels in the lung were examined. **c** mRNA levels of CD86 (M1 marker) and CD206 (M2 marker) in the lungs were examined 3 days after adoptive transfer. **d** Number of bacteria in the lungs was enumerated 3 days after adoptive transfer. Results were confirmed by 2 independent experiments. * $p < 0.05$; *** $p < 0.001$.

IFN- γ production during the acute phase of *L. pneumophila* infection, and that this affected M1/M2 polarization.

Discussion

Gr-1 was originally described as an important molecule expressed on granulocytes, which were robustly depleted by administration of the anti-Gr-1 antibody (RB6-8C5) [30, 31]. Later, several other types of inflammatory/immunological cells, including DCs and CD8⁺ T cells [19, 20], were shown to express this molecule. The anti-Gr-1 antibody recognizes the Ly6 superfamily (glycosylphosphatidylinositol-anchored membrane glycoproteins), which includes Ly6G and Ly6C. In CD3⁺CD8⁺-gated cells in lungs infected with *L. pneumophila*, the majority of cells expressed Ly6C, but not Ly6G, as well as CD44. Previously, Matsuzaki et al. [20] reported that Gr-1 antigen was expressed on memory-type CD8⁺

CD44^{high} CD62L^{high} cells and showed that anti-Gr-1 treatment greatly reduced IFN- γ production along, thereby highlighting the importance of memory-type CD8⁺ CD44^{high} CD62L^{high} cells expressing Gr-1 antigen in tumor rejection. Our findings were consistent with the findings of these reports and further demonstrated a role for IFN- γ -producing Gr-1⁺ CD8 T cells during the acute phase of resistance against *L. pneumophila* pneumonia.

NK cells are major cellular sources of IFN- γ in murine *L. pneumophila* pneumonia [32]. Sporri et al. [33] reported that NK cell-derived IFN- γ is a key cytokine involved in the resolution of *L. pneumophila* infection, and that NK cell depletion markedly increased bacterial burdens in the lungs. It was later reported that neutrophil-derived IL-18 in combination with DC-produced IL-12 triggered IFN- γ synthesis in NK cells in *L. pneumophila*-infected mice [34]. Our data demonstrated that NK cells slightly increased in the lungs during the acute phase, but anti-Gr-1 antibody treatment did not reduce the frequency of NK cells in *L. pneumophila*-infected lungs. In the present study, we reported for the first time the potential of Gr-1⁺ CD8⁺ T cells as a cellular source of IFN- γ during the acute phase of *L. pneumophila* infection in the lungs.

Regarding roles of lymphocytes in anti-*Legionella* host defense, Susa et al. [35] reported that CD8⁺ T cells were required for full clearance of *L. pneumophila* in a mouse pneumonia model, although mechanisms of anti-*Legionella* defenses, such as cellular sources and contributions of IFN- γ , were not determined. Walunas et al. [36] reported that activated CD8⁺ T cells increased the expression of Ly6C and that this also correlated with their ability to produce IFN- γ in the setting of T cell receptor/CD3 engagement induced by staphylococcal enterotoxin. They concluded that Ly6C expression correlates with an increase in IFN- γ production after antigenic stimulation of CD8⁺ T cells. Our data suggested a contribution of Ly6C⁺ CD8⁺ T cells in shifting toward the M1 state, probably through the production of IFN- γ . To this end, in our experiment involving the adoptive transfer of Ly6C⁺ CD8⁺ T cells, the protective roles of this cell type were demonstrated in the higher levels of IFN- γ , M1-directed skewing, and reduction in bacterial number in the lungs of mice undergoing adoptive transfer.

There are several limitations to the present study. Although M1-polarized macrophages exhibited relatively strong bactericidal activity against *L. pneumophila* in vitro, there are no data available describing the *L. pneu-*

mophila-targeted bactericidal activity of M1-directed macrophages in vivo. We observed a modest shift toward an M2 phenotype in the lungs of mice infected with *L. pneumophila* in the setting of anti-Gr1 antibody treatment. However, it is not clear how this M2-shift would affect *L. pneumophila* pneumonia. Several reports have suggested a correlation between M2-polarization and increased vulnerability in various organisms [37, 38]. Since one of the critical characteristics of macrophage polarization is plasticity, the M1-M2 balance may be important [26]. In addition to macrophages, other cell types and their cross-regulation in the pulmonary environment may be involved in the full protection against *L. pneumophila* pneumonia [39, 40]. Finally, our results represent only those involving a mouse model of *L. pneumophila* pneumonia. In a clinical setting, neutropenia is a known risk factor for a variety of infectious diseases, and M1/M2 status in those patients may be diverse. Our data suggested that M1/M2 status and the frequency/number of Gr-1⁺ CD8⁺ T cell might serve as markers describing the integrity of host defense systems, as demonstrated in peripheral blood monocytes.

In conclusion, this study demonstrated a shift from M1 to M2 in anti-Gr-1-treated mice with *L. pneumophila* pneumonia. Moreover, Gr-1⁺ CD8⁺ T cells were identified as a cellular source of the M1-inducing cytokine IFN- γ during the acute phase of the disease. Further studies to define the role of IFN- γ -producing Gr-1⁺ CD8⁺ T cells, especially in M1/M2-related innate immunity, are warranted to better understand macrophage-driven antibacterial mechanisms against clinically important infectious diseases.

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Author Contributions

Y.K. and C.K. designed and performed experiments and analyzed data; S.S., Y.I., Y.M., and N.I. analyzed and discussed data; T.J.S. discussed data; K.T. discussed and analyzed data.

Disclosure Statement

The authors declare no conflicts of interest associated with this work.

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