CD4CD8 Double Positive T cell responses during *Mycobacterium tuberculosis* infection in cynomolgus macaques

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1 | INTRODUCTION

In 2017, 10.0 million new cases of tuberculosis (TB) were diagnosed worldwide, with an estimated death of 1.6 million people. The host immune response plays a critical role in the outcome of *Mycobacterium tuberculosis* (Mtb) infection. Both CD4 and CD8 T cells play important roles in the immunological responses to Mtb through the production of pro-inflammatory cytokines (IFN-γ, IL-2, TNF, and IL-17), and various cytolytic functions among other mechanisms. Important roles in the CD4 T cell response to Mtb include production of IFN-γ as well as production of TNF to optimize macrophage intracellular killing. CD4 T cells also stimulate the development of cytolytic CD8 T cell functions important for Mtb killing. Cytolytic CD8 T cells (and CD4 T cells) can produce multiple cytolytic markers (e.g., granzyme B, granulysin, perforin), which can directly and indirectly kill Mtb-infected macrophages. CD8 and CD4 T cells have been studied extensively within the TB literature; however, T cells expressing both CD4 and CD8 (CD4CD8 T cells) have not been examined in the context of TB.

**Methods:** We examine peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BAL) and lung granulomas from 28 cynomolgus macaques during *Mycobacterium tuberculosis* (Mtb) infection.

**Results:** CD4CD8 T cells increase in frequency during early Mtb infection in PBMC and BAL from pre-infection. Peripheral, airway, and lung granuloma CD4CD8 T cells have distinct patterns and greater cytokine production than CD4 or CD8 T cells.

**Conclusion:** Our data suggest that CD4CD8 T cells transient the blood and airways early during infection to reach the granulomas where they are involved directly in the host response to Mtb.

**Keywords**
CD4CD8, mycobacteria, T cells, tuberculosis

Abstract

**Background:** Tuberculosis (TB) kills millions of people every year. CD4 and CD8 T cells are critical in the immune response against TB. T cells expressing both CD4 and CD8 (CD4CD8 T cells) are functionally active and have not been examined in the context of TB.

**Methods:** We examine peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BAL) and lung granulomas from 28 cynomolgus macaques during *Mycobacterium tuberculosis* (Mtb) infection.

**Results:** CD4CD8 T cells increase in frequency during early Mtb infection in PBMC and BAL from pre-infection. Peripheral, airway, and lung granuloma CD4CD8 T cells have distinct patterns and greater cytokine production than CD4 or CD8 T cells.

**Conclusion:** Our data suggest that CD4CD8 T cells transient the blood and airways early during infection to reach the granulomas where they are involved directly in the host response to Mtb.

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functionally respond to HIV, SHIV, Mycobacterium leprae, and various cancers. CD4CD8 T cells from adults with rheumatoid arthritis produce more IFN-γ than peripheral CD4 and CD8 T cells stimulated with mitogens. Taken together, CD4CD8 T cells express both pro-inflammatory and anti-inflammatory cytokines production and are able to exhibit cytolytic function. However, they have not been examined in the context of TB.

Here, we sought to characterize these CD4CD8 T cells during the course of Mtb infection in the blood, airways, and lung granulomas using a cynomolgus macaque model of TB. We show that CD4CD8 T cells increase in frequency in both blood and the airways during early Mtb infection and within lung granulomas with Mtb-specific responses that are similar but distinct in pattern from conventional single-expressing CD4 and CD8 T cells.

2 | METHODS

2.1 | Animals

Adult (>4 years of age) cynomolgus macaques (Macaca fascicularis) were screened for other co-morbidities (e.g. parasites, SIV) before infection (Valley Biosystems, Sacramento, CA). Animals were infected with low-dose (~25 CFU per monkey) Mtb (Erdman strain) via bronchoscopic instillation to the lower lung lobe and housed in Biosafety Level 3 (BSL-3) primate facility. Infection was confirmed as described previously. Animals were declared as having active disease (i.e. repeated presence of Mtb growth in bronchoalveolar lavage [BAL] or gastric aspirate, elevated sedimentation rate [ESR], and/or clinical signs of active TB after infection) or latent infection (i.e. no clinical signs of active TB, normal ESR, and lack of persistent Mtb growth by 6 months post-infection) based on previously published criteria. Twenty-four monkeys were included in the PBMC intracellular cytokine (ICS) analysis, 28 monkeys were included in the BAL profile, and eight monkeys were included in the lung granuloma profile. Due to restrictions on animal procedures or low cell counts, some time points use fewer monkeys. Immune data for eight of these animals were previously published but without CD4CD8 T cell analysis. All animal protocols and procedures were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee that adheres to the national guidelines established in the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals as mandated by the U.S. Public Health Service Policy.

2.2 | Procedures

Cells from the BAL and PBMCs were obtained for analysis prior to Mtb infection, 4 weeks, 12 weeks post-infection, and at time of clinical diagnosis (latent or active TB) as previously described. Isolated cells were stimulated with ESAT6-CFP10 peptide pools (10 μg/mL of every peptide) or incubated with only media (RPMI+10%shAB). PBMC incubated with or without the peptide pools for 1 hour in media, followed by the addition of Brefeldin A (Golgiplug: BD biosciences) for 5 hours at 37°C with 5% CO₂. ESAT6-CFP10 responses within PBMC T cells are presented after subtracting responses from PBMC incubated with media. BAL and granuloma cells were isolated and stimulated for 4 hours with peptide pools in the presence of Brefeldin A. When used, CD107a antibody was added prior to incubation. A minimum of 5 × 10⁵ BAL or PBMC cells were incubated with each stimulation condition and acquired.

A total of 29 granulomas were used for analysis from eight macaques (2-5 granulomas per animal) using the same ICS protocol as above for BAL. The range of cells analyzed from granulomas included CD4 (median = 116, IQR25%-75% = 80-347), CD8 (median = 135, IQR25%-75% = 78-304), and CD4CD8 (median = 111, IQR25%-75% = 74-260). T cells were in the estimated range of a previously published threshold. Granuloma-specific T cell responses were only available from clinically latent animals as animals with active TB were dedicated for other studies and immune data were not available.

2.3 | Flow cytometry

Flow cytometry was performed on PBMC, BAL, and granuloma cells after stimulation and stained with a combination of antibodies (Table S1), as previously described. PBMC were stained for 2 panels: cytokines (CD3, CD4, CD8, IFN-γ, TNF, IL-2, IL-10, and IL-17) or cytolytic markers (CD107a, granzyme B, granulysin, perforin). BAL cells were stained for CD3, CD4, CD8, IFN-γ, TNF, IL-2, IL-10, and IL-17. Granuloma cells were stained for CD3, CD4, CD8, IFN-γ, TNF, IL-2, IL-10, IL-17, and granzyme B. Data acquisition was performed using an LSR II (BD) and analyzed using FlowJo Software v.9.7 (Treestar Inc, Ashland, OR). Gating strategies used for analysis are presented using ESAT6-CFP10-stimulated PBMC (Figure S1). To eliminate the possibility that CD4CD8 T cell population is a result of fluorophore spillover, the population was confirmed using combinations of fluorophores that are excited by different lasers (Figure S1).

2.4 | Statistical analysis

Data were tested for normality with the Shapiro-Wilk test and were not consistently normally distributed, so all statistics utilized were non-parametric analyses (JMP Pro 12.1.0 statistical software). Kruskal-Wallis test with Dunn’s multiple comparison test was used to compare changes in cell type, cytokine production, and cytolytic marker expression over time within BAL and PBMC (GraphPad Prism 7.0d statistical software). Wilcoxon method (with unadjusted P-values) was used to compare frequencies of CD4, CD8, and CD4CD8 T cells in PBMC and BAL and granulomas within the same time points (JMP Pro 12.1.0 statistical software). Statistical significance was set at P ≤ 0.05. All P values P ≤ 0.1 are displayed to represent statistical trends.

3 | RESULTS

To characterize the dynamics in CD4CD8 T cells during the course of Mtb infection, the frequency and function of CD4CD8 T cells...
in PBMC and BAL were examined before *M. tuberculosis* infection, 4 weeks, 12 weeks post-infection, and at the time of latent infection diagnosis (at 6 months after infection) and active TB diagnosis (typically before 6 months after infection) (Figure 1). CD4CD8 T cell frequencies doubled from pre-infection levels at 4 weeks post-Mtb infection (Figure 1) in both PBMC and BAL cells. At 4 weeks post-Mtb infection, an increased production of Mtb-specific TNF and granzyme B was observed in PBMC CD4CD8 T cells compared to pre-infection levels (Figure 1). Similarly, CD4CD8 T cells in the airways increased significantly at 4 weeks post-Mtb infection compared to pre-infection levels. Higher frequencies of TNF producing airway CD4CD8 T cells were also seen at 4 weeks after Mtb infection but then decreased to pre-infection levels during the remaining infection period.

To distinguish PBMC and BAL CD4CD8 T cells from CD4 or CD8 T cells, we compared the frequency and activity of each cell type within the same time point during infection (Tables 1 and 2). In blood, a significantly smaller frequency of CD4CD8 T cells was observed at all time points compared to CD4 and CD8 T cells. Within PBMC, CD4CD8 T cells are able to produce both cytolytic markers and Th1 cytokines during acute TB. At 4 weeks post-Mtb infection, Mtb-specific TNF and granzyme B was observed in PBMC CD4CD8 T cells compared to pre-infection levels (Figure 1). Similarly, CD4CD8 T cells in the airways increased significantly at 4 weeks post-Mtb infection compared to pre-infection levels. Higher frequencies of TNF producing airway CD4CD8 T cells were also seen at 4 weeks after Mtb infection but then decreased to pre-infection levels during the remaining infection period.

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| PBMC | Median [IQR 25%, 75%] | Median [25%, 75%] | Median [25%, 75%] | Median [25%, 75%] | Median [25%, 75%] |
|------|------------------------|-------------------|-------------------|-------------------|-------------------|
| T cell % | 0 | 52.9 [40.0-58.4] | 3.2 [0.77-4.4] | 35.6 [30.1-41.6] | <0.0001 |
| | 4 wk | 46.7 [36.6-54.9] | 4.4 [3.1-7.5] | 40.0 [31.9-47.0] | <0.0001 |
| | 12 wk | 45.4 [36.5-54.3] | 3.8 [0.80-9.9] | 43.3 [34.3-51.5] | <0.0001 |
| | Dx | 52.5 [40.0-58.8] | 3.9 [1.2-8.1] | 38.3 [31.1-45.8] | <0.0001 |
| CD107a % | 0 | 0.05 [0-0.2] | 0.081 [0-0.50] | 0.10 [0.02-0.42] | 0.0570 |
| | 4 wk | 0.015 [0-0.56] | 0.10 [0-0.43] | 0.25 [0.07-0.42] | 0.0570 |
| | 12 wk | 0.028 [0-0.10] | 0.10 [0-0.20] | 0.11 [0.01-0.44] | 0.0570 |
| | Dx | 0 [0-0.12] | 0.19 [0-1.3] | 0 [0-0.2] | 0.0722 |
| Granulysin % | 0 | 0.08 [0-0.45] | 0.4 [0-1.5] | 0.4 [0.04-1.6] | 0.0288 |
| | 4 wk | 0.2 [0-0.56] | 0.7 [0-1.9] | 0.9 [0.3-3.3] | 0.0288 |
| | 12 wk | 0.1 [0-0.42] | 0.3 [0-0.56] | 0.4 [0-1.0] | 0.0288 |
| | Dx | 0.067 [0-1.1] | 0 [0-1.4] | 0.065 [0-3.2] | 0.0288 |
| Granzyme B % | 0 | 0.14 [0-1.4] | 0.49 [0-4.6] | 0.24 [0-3.5] | 0.0434 |
| | 4 wk | 0.8 [0.1-2.9] | 5.2 [0.9-13.9] | 2.1 [0.2-15.2] | 0.0207 |
| | 12 wk | 3.0 [0.03-5.2] | 3.4 [0-12.7] | 1.4 [0-18.1] | 0.0207 |
| | Dx | 0 [0-4.0] | 0 [0-4.5] | 0.3 [0-4.0] | 0.0207 |
| Perforin % | 0 | 0 [0-0.05] | 0.19 [0-0.97] | 0.3 [0.01-1.4] | 0.0288 |
| | 4 wk | 0.06 [0.02-0.45] | 1.04 [0.5-2.1] | 0.69 [0.3-2.4] | 0.0434 |
| | 12 wk | 0.02 [0-0.09] | 0.1 [0-0.42] | 0.23 [0.1-0.51] | 0.0434 |
| | Dx | 0 [0-0.2] | 0 [0-0.47] | 0.023 [0-0.83] | 0.0434 |
| IL10% | 0 | 0.01 [0-0.2] | 0.40 [0-1.3] | 0.01 [0-0.1] | 0.0434 |
| | 4 wk | 0.7 [0-3.5] | 0 [0-0.9] | 0 [0-0.5] | 0.0434 |
| | 12 wk | 0.012 [0-3.5] | 0 [0-0.8] | 0.11 [0-0.3] | 0.0434 |
| | Dx | 0.52 [0-1.3] | 0.29 [0-1.6] | 0.53 [0-4.4] | 0.0434 |
| IFN-γ % | 0 | 0.03 [0-0.13] | 0 [0-0.72] | 0.03 [0-0.14] | 0.0434 |
| | 4 wk | 0.15 [0.02-0.34] | 0.25 [0-0.75] | 0.05 [0-0.16] | 0.0434 |
| | 12 wk | 0.10 [0-0.30] | 0.07 [0-0.40] | 0.06 [0-0.14] | 0.0434 |
| | Dx | 0.02 [0-1.3] | 0.07 [0-5.0] | 0 [0-1.4] | 0.0434 |
| IL2% | 0 | 0.093 [0-0.15] | 0.27 [0-0.5] | 0.11 [0.02-0.2] | 0.0434 |
| | 4 wk | 0.13 [0-0.32] | 0.002 [0-1.5] | 0.11 [0.02-0.36] | 0.0434 |
| | 12 wk | 0.09 [0-0.34] | 0.42 [0-0.76] | 0.083 [0-0.27] | 0.0434 |
| | Dx | 1.2 [0.2-2.9] | 0 [0-0.01] | 1.1 [0-3.7] | <0.0001 |
| TNF % | 0 | 0.045 [0-0.12] | 0.015 [0-0.18] | 0.037 [0-0.08] | 0.0434 |
| | 4 wk | 0.36 [0.06-0.47] | 0.49 [0-1.1] | 0.12 [0-0.33] | 0.0434 |
| | 12 wk | 0.20 [0-1.4] | 0.33 [0-1.1] | 0.16 [0-0.2] | 0.0434 |
| | Dx | 0.47 [0.02-1.2] | 0.41 [0-1.7] | 0 [0-0.84] | 0.0434 |
| IL17% | 0 | 0 [0-0.08] | 0 [0-0] | 0 [0-0.17] | 0.0434 |
| | 4 wk | 0.10 [0-0.76] | 0.13 [0-1.6] | 0.015 [0-0.24] | 0.0434 |
| | 12 wk | 0.014 [0-0.22] | 0.03 [0-0.58] | 0.0043 [0-0.2] | 0.0434 |
| | Dx | 0.76 [0.15-2.8] | 1 [0-3.5] | 0.018 [0-1.9] | 0.0434 |

CD4, CD4CD8, and CD8 T cells within the T cell gate (CD3+) are shown in the top row. Percentages of cells expressing each cytolytic marker or cytokine are represented for CD4, CD4CD8, or CD8 T cells. ESAT6/CFP10 stimulated cells were subtracted from media incubated cells. Analysis performed across each cell type within each time point using the (unadjusted P-values shown in last column).

Dx, time that clinical outcome is declared (active disease or latent infection); Wk, weeks post-infection. Significance is P ≤ 0.05.
T cell responses in the granuloma are known to play a key role in outcome; therefore, we sought to characterize the frequency and functionality of CD4CD8 T cells in granulomas (Figure 2) and compare them to single positive CD4 and CD8 T cells. CD4CD8 T cell accounted for a median of 11% of CD3 T cells in the granuloma, and this was significantly less than the 36.2% CD4 T cells and 25% CD8 T cells (Figure 2A). A significantly lower frequency of CD4CD8 T cells expressed granzyme B compared to both CD4 and CD8 T cells (Figure 2A). The proportion of CD4CD8 T cells producing IL-10 was greater than CD8 T cells but similar to CD4 T cells. In contrast, the proportion of TNF producing CD4CD8 T cells was significantly higher than CD4 T cells and more similar to CD8 T cells. No differences were observed in their production of IFN-γ, IL-2, or IL-17 compared to conventional CD4 and CD8 T cells. These data demonstrate that CD4CD8 T cells have both distinct and similar functions from CD4 and CD8 T cells within individual granulomas.

In summary, CD4CD8 T cells are increased in frequency during early Mtb infection in the blood and airways and can be found in granulomas with distinct Mtb-specific functions from single-expressing CD4 and CD8 T cells.

4 | DISCUSSION

This study aimed to characterize the frequency and function of CD4CD8 T cells during Mtb infection in PBMC, BAL, and lung granulomas and determine if they are functionally distinct from CD4 and CD8 T cells. CD4CD8 T cells are traditionally regarded as existing during a time of cellular differentiation toward CD4 or CD8 T cells. However, extrathymic CD4CD8 T cells located within the blood and numerous tissues have been reported and more than 96% of blood CD4CD8 T cells express alpha-beta TCR.

### TABLE 2 Frequency of T cells from bronchoalveolar lavage cells producing cytokines during Mycobacterium tuberculosis infection in non-human primates (n = 28). CD4, CD4CD8, and CD8 T cells within the T cell gate (CD3+) are shown in the top row.

| BAL   | Weeks post-Mtb infection | CD4 Median [25%, 75%] | CD4CD8 Median [25%, 75%] | CD8 Median [25%, 75%] | P value (CD4 vs CD4CD8) | P value (CD8 vs CD4CD8) |
|-------|--------------------------|------------------------|---------------------------|------------------------|--------------------------|--------------------------|
| T cell % | 0                        | 36.8 [31.4-45.6]       | 4.0 [2.5-6.2]             | 39.7 [31.3-46.1]       | <0.0001                  | <0.0001                  |
|       | 4 wk                     | 30.5 [22.4-42.0]       | 8.0 [4.1-15.3]            | 42 [40.5-49.6]         | <0.0001                  | <0.0001                  |
|       | 12 wk                    | 37.4 [23.0-45.2]       | 3.9 [2.5-12.6]            | 44.6 [39.9-52.5]       | <0.0001                  | <0.0001                  |
|       | Dx                       | 33.4 [26.6-44.3]       | 2.9 [1.2-9.2]             | 50.2 [40.8-56.6]       | <0.0001                  | <0.0001                  |
| IFN-γ % | 0                        | 0.75 [0.4-2.2]         | 6.5 [0.6-16.0]            | 2.1 [0.7-5.5]          | 0.0027                   | 0.2322                   |
|       | 4 wk                     | 2.9 [1.1-9.4]          | 5.3 [2.8-9.6]             | 1.4 [0.5-2.5]          | 0.0753                   | <0.0001                  |
|       | 12 wk                    | 2.9 [0.98-6.3]         | 3.4 [1.4-5.6]             | 0.85 [0.5-2.2]         | 0.0001                   |                          |
|       | Dx                       | 1.45 [0.5-4.1]         | 1.2 [0.3-2.8]             | 0.3 [0.1-0.6]          | 0.0126                   |                          |
| IL-10% | 0                        | 2.3 [0.9-7.3]          | 8.1 [3.3-13.6]            | 2.5 [1.1-3.7]          | 0.0192                   | 0.0020                   |
|       | 4 wk                     | 1 [0.2-3.7]            | 6.1 [2.2-13.2]            | 1.1 [0.38-4.3]         | 0.0284                   | 0.0365                   |
|       | 12 wk                    | 1.9 [0.6-5.4]          | 9.7 [4.9-19.2]            | 2.4 [0.5-13.6]         | 0.0015                   |                          |
|       | Dx                       | 0.66 [0.2-3.2]         | 7.1 [0.3-16.0]            | 1.7 [0.05-6.5]         |                          |                          |
| IL-17% | 0                        | 1.4 [0.7-3.1]          | 5.6 [2.6-14.6]            | 1.25 [0.6-2.6]         | <0.0001                  | <0.0001                  |
|       | 4 wk                     | 2.4 [1.2-7.9]          | 5.3 [2.4-10.4]            | 0.95 [0.5-3.7]         | 0.0464                   | 0.0016                   |
|       | 12 wk                    | 0.8 [0.3-2.6]          | 2.8 [1.6-7.7]             | 0.3 [0.1-0.58]         | 0.0008                   | <0.0001                  |
|       | Dx                       | 0.34 [0-1.9]           | 4.76 [1.7-9.9]            | 0.35 [0.1-0.65]        | <0.0001                  | <0.0001                  |
| IL-2%  | 0                        | 1.3 [0.8-5.5]          | 5.1 [2.3-9.6]             | 1.3 [0.8-2.6]          | 0.0046                   | 0.0001                   |
|       | 4 wk                     | 3.6 [1.5-5.2]          | 4.7 [3.3-7.0]             | 1.6 [0.9-2.1]          | 0.0003                   |                          |
|       | 12 wk                    | 2.3 [1.4-3.4]          | 3.4 [2.4-5.1]             | 0.8 [0.5-1.7]          | 0.0460                   | <0.0001                  |
|       | Dx                       | 2.1 [1.0-3.2]          | 2.4 [0.9-5.8]             | 1.1 [0.2-1.7]          | 0.0236                   |                          |
| TNF %  | 0                        | 1.5 [0.9-4]            | 6.5 [1.6-10.7]            | 2.8 [1.5-7.8]          | <0.0051                  |                          |
|       | 4 wk                     | 6.8 [3-12.3]           | 10.0 [4.1-19.4]           | 2.6 [0.9-9.2]          | 0.0018                   |                          |
|       | 12 wk                    | 5.1 [2.5-8.1]          | 5.2 [3.2-7.4]             | 2 [1.0-3.0]            | 0.0001                   |                          |
|       | Dx                       | 1.8 [0.2-5.2]          | 0.76 [0.4-5.3]            | 1.2 [0.2-3.2]          |                          |                          |

Percentages of cells expressing each cytokine are represented for CD4, CD4CD8, or CD8 T cells from cells stimulated with ESAT6/CFP10. Analysis performed on each pair of cell types at each time point using the Wilcoxon method (unadjusted P-values shown in last column). Dx, time that clinical outcome is declared (active disease or latent infection); Wk, weeks post-infection. Significance is P ≤ 0.05.
We determined that CD4CD8 T cells represent a minority population of T cells within PBMC (median ranges pre- and 4 week post-infection of 2.2%-4.4%), BAL (median ranges pre- and 4 weeks post-infection of 2.9%-8.0%), and lung granulomas (median 11%). In blood, Mtb-specific CD4CD8 T cells express more cytolytic markers (CD107a, granzyme B, and perforin) than CD4 and CD8 T cells. C and D) Differences in TNF and IL-10 production in CD4CD8 T cells compared to CD4 and CD8 T cells. Each data point represents an individual lung granulomas. Different symbols represent individual macaques. Kruskal-Wallis test with Dunn’s multiple comparison (post hoc), P-values shown.

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CONFLICTS OF INTEREST
The authors do not have any conflicts of interest associated with this manuscript.

AUTHOR CONTRIBUTION
Conception and design: CRD, PLL; Analysis and interpretation: CRD, TR, AJM, HPG, PLL, PM; Experimentation: CRD, TB, TR, AJM, HPG.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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