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Cutting Edge: Direct Recognition of Infected Cells by CD4 T Cells Is Required for Control of Intracellular Mycobacterium tuberculosis In Vivo

Smita Srivastava* and Joel D. Ernst*†‡

Effector T cells control intracellular infection by secreting cytokines and through contact-dependent cytolyis. Because cytokines can diffuse and act at a distance, we determined whether cytokine diffusion is sufficient to control Mycobacterium tuberculosis or whether direct recognition of infected cells by CD4 T cells is required. Using MHC class II (MHC II) mixed bone marrow chimeras, we compared the bacterial burdens in lung myeloid cells that were capable (MHC II+/+) or not (MHC II−/−) of being recognized by CD4 T cells. MHC II+/+ cells had lower bacterial burdens than did MHC II−/− cells. CD4 T cell depletion increased the number of bacteria associated with MHC II+/+ cells but not MHC II−/− cells, indicating that direct recognition of infected cells by CD4 T cells is required for control of intracellular M. tuberculosis. These results show that the effector mechanisms required for CD4 T cell control of distinct intracellular pathogens differ and that long-range cytokine diffusion does not contribute to control of M. tuberculosis. The Journal of Immunology, 2013, 191: 1016–1020.

CD4 and CD8 T cells contribute to immunity through cytolytic activity and by secretion of cytokines. Cytolytic activity requires direct recognition of an infected cell and formation of an immunological synapse, the site of Ag-specific CTL activation, as well as the site of delivery of cytolytic effector molecules that kill the target cell (1, 2). Cytokine-secreting CD4 and CD8 T cells also require activation through immunological synapses, followed by vectorial or multidirectional release of cytokines (3). Secreted cytokines may act directly on the infected cell bearing the peptide-MHC complexes that provide the signal for T cell activation (3, 4), or they may diffuse within a tissue to activate “bystander” cells, which were not involved in engaging and activating Ag-specific T cells (5, 6). A recent study reported that Leishmania major, an intracellular pathogen of macrophages and dendritic cells (DC), can be controlled by bystander activation of Ag-specific CD4 T cells through secretion and diffusion of IFN-γ, which caused killing of intracellular L. major up to 80 μm from the activated T cell (5).

Mycobacterium tuberculosis is also an intracellular pathogen of macrophages and DC, and it requires CD4 T cells and IFN-γ to restrict its growth and dissemination (7). Unlike L. major, M. tuberculosis can persist and cause disease with high pathogen burdens, despite the expansion, differentiation, and trafficking of CD4 T cells to the site of infection (8). However, it remains unclear precisely how CD4 T cells contribute to the control of M. tuberculosis (9). In mice, administration of Th1-polarized TCR-transgenic CD4 T cells specific for the M. tuberculosis Ag, ESAT-6, provides potent antitycrobacterial effects (10), yet these effects are independent of the ability of the transferred T cells to produce either IFN-γ or TNF (11). Similarly, reconstitution of RAG2−/− mice by transfer of CD4 memory T cells from mice previously infected with M. tuberculosis conferred comparable control of subsequent infection, whether the transferred T cells were competent to produce IFN-γ or not (12).

In humans, the mechanisms used by CD4 T cells to control the progression of M. tuberculosis are similarly poorly understood. In a recent clinical trial of bacillus Calmette-Guérin vaccination, infants that were protected from tuberculosis (TB) did not differ with regard to their mycobacterial Ag-induced CD4 or CD8 T cell secretion of IFN-γ, TNF, IL-2, or IL-17 from infants that developed TB (13). Likewise, adults can exhibit Ag-specific polyfunctional CD4 and CD8 T cell responses, yet still progress to active TB (14–16). Therefore, although CD4 T cells are essential for immunity to M. tuberculosis, the effector mechanisms that they use to control M. tuberculosis are incompletely defined.

To further understand the contributions of CD4 T cells to immunity in TB, we determined whether immune control of M. tuberculosis requires direct recognition of infected cells or whether bystander activation of CD4 effector cells is sufficient to restrict infection.

Materials and Methods

Generation and infection of MHC class II mixed bone marrow–chimeric mice and CD4 T cell depletion

Mixed bone marrow chimeras were generated as described (17), using T cell–depleted MHC class II (MHC II)−/− (CD45.1−) and MHC II+/+ (CD45.2+) mice. Reconstituted recipient mice were infected with M. tuberculosis H37Rv. Depletion of CD4 T cells was achieved by depletion of CD4+ T cells from donor mice prior to engraftment as described (17). This work was supported by National Institutes of Health Grants R01 AI051242 and R01 AI084041.
bone marrow cells. Chimeras were infected 7 wk later by aerosol (∼100 CFU/mouse) with \(M.\) \(tuberculosis\) H37Rv that expresses FACS-optimized GFP (18, 19). Selected animals were treated every 5 d with 500 μg CD4-depleting Ab (GK1.5) or isotype control (LTF-2), starting at 18 d postinfection and continuing until the day of harvest (day 35). Single-cell intracellular bacterial burdens were determined in sorted GFP+ cell subsets by fluorescence microscopy and manual counting. Bacterial loads in lung homogenates and sorted cell populations were determined by plating serial dilutions on 7H11 agar. Procedures involving mice were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

**Cell sorting**

Lung cells from MHC II mixed chimeras infected with \(M.\) \(tuberculosis\) H37Rv-GFP were stained with CD11c, CD11b, and Gr-1 Abs, as described (19, 20). Following staining, samples were pooled (two to four mice/pool; three pools/group), and live cells were sorted using an iCyt Synergy sorter in BSL-3 containment into MHC II+/+ and MHC II−/− CD11chiCD11bhi DC and CD11clo/intCD11bmed recruited macrophages (RM). GFP+ cells were sorted from each of the DC and RM subsets and either fixed for microscopic determination of single-cell bacterial burdens or plated for quantitation of live bacteria.

**Statistical analysis**

Statistical comparisons were performed with Prism 4 for Macintosh (GraphPad, San Diego, CA), using the tests specified in the figure legends. The \(p\) values \(< 0.05\) were considered significant.

**Results and Discussion**

To determine whether direct recognition of infected cells by CD4 effector T cells is required for optimal control of \(M.\) \(tuberculosis\), we prepared mixed bone marrow–chimeric mice using equal numbers of cells from MHC II+/+ (CD45.1) and MHC II−/− (CD45.2) mice and infected them with \(M.\) \(tuberculosis\) that expresses FACS-optimized GFP (17–19). This allows assessment of the number of infected cells and the number of bacteria associated with cells that can (MHC II+/+) or cannot (MHC II−/−) be directly recognized by CD4 T cells, after isolation from the same lung environment.

Following infection with \(M.\) \(tuberculosis\), mice reconstituted with 50% MHC II+/+ and 50% MHC II−/− bone marrow responded with equivalent (by frequency; Fig. 1A) or slightly greater (by cell number; Fig. 1B) CD4 T cell recruitment to the lungs compared with mice reconstituted with 100% MHC II+/+ marrow, indicating that MHC II+/+ cells were present in sufficient numbers in the II+/+−/− mixed chimeras to accomplish initial priming and trafficking of CD4 T cells to the lungs. Despite the presence of CD4 T cells at equivalent frequencies in the lungs of the two groups of mice, IFN-γ concentrations were ∼20% lower in lung homogenates from the II+/+−/− mixed chimeras (Fig. 1C), consistent with less effector T cell activation in the lungs. Concordant with the evidence of less effector T cell activation, the II+/+−/− mice had 5-fold more bacteria in the lungs on day 35 postinfection (Fig. 1D).

To determine whether the higher bacterial burdens in the lungs of II+/+−/− chimeras were attributable to the presence of bacteria in a larger proportion of MHC II−/− cells than MHC II+/+ cells, we took advantage of bacterial GFP expression to identify and quantitate infected lung leukocytes by flow cytometry. We concentrated on two cell subsets that are the most frequently infected in the lungs after the onset of adaptive immunity: CD11chiCD11bhi DC and CD11clo/intCD11bmed RM (19). This revealed that 8–11% of the cells in each subset contained bacteria, as determined by flow cytometry after isolation from the lungs of mixed chimeras (Fig. 2); this did not differ significantly by the presence or absence of MHC II in either subset (DC: II+/+ 9.1 ± 0.8, II−/− 10.8 ± 0.9, \(p = 0.4\); RM: II+/+ 8.2 ± 0.5, II−/− 11 ± 1.1, \(p = 0.1\)). These results indicate that the absence of MHC II expression on lung DC or RM has little or no effect on the proportion of cells that contain bacteria.

As an alternative explanation for the larger bacterial burden in the lungs of II+/+−/− mixed chimeras, we determined whether the absence of MHC II expression resulted in poorer control of infection manifest as a larger number of bacteria/infected cell. We sorted lung cells from \(M.\) \(tuberculosis\)-GFP–infected MHC II mixed chimeras on the basis of cell phenotype (DC or RM), the presence or absence of the ability to express MHC II (using CD45.1+ to identify MHC II+/+ and CD45.1− to identify MHC II−/− cells [from CD45.2 donors]), and the presence of GFP fluorescence. The number of bacteria/cell in GFP+ DC and RM was then quantitated by fluorescence microscopy. This revealed that the number of bacteria/infected DC (Fig. 3A) or RM (Fig. 3B) was greater in MHC II−/− cells than in MHC II+/+ cells of either myeloid phenotype. Because the absence of MHC II may have effects other than the loss of CD4 T cell recognition (21), we depleted CD4 T cells from the II+/+−/− mixed chimeras and found that this increased the total lung bacterial burden (Fig. 1D), increased the number of GFP+ bacteria/infected MHC

**FIGURE 1.** CD4 T cells, IFN-γ, and bacterial burdens in mice reconstituted with MHC II+/+ or mixed MHC II+/+ and MHC II−/− bone marrow and infected with \(M.\) \(tuberculosis\). Irradiated MHC II wild-type (CD45.1) mice were reconstituted with either 100% MHC II wild-type (II+/+−/−) (CD45.1−) or a 1:1 mixture of MHC II+/+ (CD45.1+) and MHC II−/− (CD45.2−) bone marrow cells (II+/+−/−) and infected with \(M.\) \(tuberculosis\) H37Rv expressing GFP (100 CFU/mouse) 7 wk later. A group of II+/+−/− chimeras was depleted of CD4 T cells beginning on day 18 postinfection. (A) Frequency of CD4 T cells in lungs of mice reconstituted with wild-type (II+/+−/−) or mixed II+/+−/− bone marrow. (B) Total number of CD4 T cells in lungs of II+/+−/− and II+/+−/−−/− chimeras. (C) IFN-γ in lung homogenates from mice reconstituted with wild-type (II+/+−/−) or mixed (II+/+−/−−/−) bone marrow. (D) Effect of CD4 T cell depletion on lung bacterial burdens in II+/+−/− and II+/+−/−−/− bone marrow chimeras. Data represent four to six mouse replicates. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). Student t test. n.s., Not significant.
II+ cell, and abolished the difference in the frequency distribution of bacteria in MHC II+/+ cells compared with MHC II−/− cells (Fig. 3A, 3B, right panels). These results indicate that MHC II+/+ DC and RM control intracellular M. tuberculosis more effectively than do MHC II−/− cells, and this difference is CD4 T cell dependent. This suggests that direct recognition of infected DC or RM by CD4 T cells is necessary for optimal control of M. tuberculosis.

Because GFP+ intracellular bacteria could include both live and dead bacteria, we determined whether the larger number of GFP+ bacteria associated with MHC II−/− DC and RM observed by microscopy reflected a larger number of live
bacteria. Using the same sorting parameters as described for quantitating bacteria/infected cell by microscopy, we quantitated live bacteria (as CFU)/1000 GFP+ cells in each sorted fraction. This revealed that the number of live \textit{M. tuberculosis} is greater in MHC II+/− DC and RM compared with MHC II+/+ DC and RM in CD4 T cell–replete mice (Fig. 3C, 3D). Depletion of CD4 T cells resulted in significant increases in the number of live bacteria associated with MHC II+/+ DC and RM, confirming that CD4 T cells contribute to the control of intracellular \textit{M. tuberculosis} by restricting the number of bacteria/infected cell. In contrast, CD4 T cell depletion had no effect on the number of live bacteria associated with MHC II+/+ DC or RM (Fig. 3C, 3D). This indicates that CD4 T cell control of intracellular \textit{M. tuberculosis} depends on direct recognition of infected cells and that this mechanism predominates over those that can occur as the consequence of bystander activation, such as through the action of diffusible cytokines.

The requirement for direct recognition of infected lung myeloid cells by CD4 effector T cells for optimal control of \textit{M. tuberculosis} is clearly distinct from findings with regard to \textit{L. major}, in which bystander activation of CD4 effector cells is sufficient for control of intracellular parasites (5). There are three possible explanations for the differences in the results with \textit{L. major} and \textit{M. tuberculosis}. One is that infection with \textit{M. tuberculosis} establishes a tissue environment in which diffusion of cytokines secreted by activated CD4 effector cells is restricted, thereby preventing secreted cytokines from acting on infected cells at a distance. For example, IFN-γ binds to glycosaminoglycans, restricting the diffusion and the action of IFN-γ (22), and glycosaminoglycans are deposited in high concentrations at the site of infection in TB (23). Related to this, another possibility is that the amount of IFN-γ produced per CD4 effector cell is low in TB, and this restricts its effects to adjacent cells. The other potential explanation for the requirement for direct CD4 T cell recognition of \textit{M. tuberculosis}–infected cells in vivo is that control of \textit{M. tuberculosis} requires the action of one or more mechanisms that require direct contact between CD4 effector cells and \textit{M. tuberculosis}–infected cells. This is consistent with the recent observations that CD4 T cells contribute to the control of \textit{M. tuberculosis} in vivo, even when they are not competent to secrete IFN-γ or TNF, express perforin, or interact with Fas (11, 12). Our results suggest that further investigation of CD4 effector mechanisms in TB must concentrate on mechanisms that require direct recognition and contact with infected cells.

Our results have implications for TB vaccine development. First, these results suggest that TB vaccines that promote the development of CD4 T cell mechanisms that operate through direct recognition and contact of infected cells may have greater efficacy than do vaccines that induce multifunctional cytokine responses alone. Therefore, investigation of vaccine vectors and adjuvants should include the determination of their ability to induce T cells with effector mechanisms that extend beyond cytokine secretion. Second, considering recent evidence that the frequency of Ag-specific CD4 effector T cell activation at the site of infection is low (4, 24) and that this limits the antimycobacterial efficacy of Ag-specific CD4 effector T cells (24), the results reported in this article indicate the need for better understanding of the mechanisms of Ag presentation and their limitations in cells infected with \textit{M. tuberculosis}. In light of evidence that \textit{M. tuberculosis} interferes with MHC II Ag presentation by the cells that it infects (25–30), vaccine-induced CD4 T cells may have limited efficacy unless the mechanism(s) that limit Ag presentation can be overcome or bypassed. The evidence that optimal control of intracellular \textit{M. tuberculosis} requires direct recognition of infected cells by CD4 effector T cells heightens the importance of identifying the mechanisms that attenuate MHC II Ag presentation in \textit{M. tuberculosis}–infected cells.

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Disclosures
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