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This information is current as of July 14, 2017.

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*J Immunol* 2001; 167:6991-7000; ;  
doi: 10.4049/jimmunol.167.12.6991  
<http://www.jimmunol.org/content/167/12/6991>

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# CD4<sup>+</sup> T Cells Are Required for the Development of Cytotoxic CD8<sup>+</sup> T Cells During *Mycobacterium tuberculosis* Infection<sup>1</sup>

Natalya V. Serbina,<sup>2</sup> Vanja Lazarevic, and JoAnne L. Flynn<sup>3</sup>

The control of acute and chronic *Mycobacterium tuberculosis* infection is dependent on CD4<sup>+</sup> T cells. In a variety of systems CD8<sup>+</sup> T cell effector responses are dependent on CD4<sup>+</sup> T cell help. The development of CD8<sup>+</sup> T cell-mediated immune responses in the absence of CD4<sup>+</sup> T cells was investigated in a murine model of acute tuberculosis. In vitro and in vivo, priming of mycobacteria-specific CD8<sup>+</sup> T cells was unaffected by the absence of CD4<sup>+</sup> T cells. Infiltration of CD8<sup>+</sup> T cells into infected lungs of CD4<sup>-/-</sup> or wild-type mice was similar. IFN- $\gamma$  production by lung CD8<sup>+</sup> T cells in CD4<sup>-/-</sup> and wild-type mice was also comparable, suggesting that emergence of IFN- $\gamma$ -producing mycobacteria-specific CD8<sup>+</sup> T cells in the lungs was independent of CD4<sup>+</sup> T cell help. In contrast, cytotoxic activity of CD8<sup>+</sup> T cells from lungs of *M. tuberculosis*-infected mice was impaired in CD4<sup>-/-</sup> mice. Expression of mRNA for IL-2 and IL-15, cytokines critical for the development of cytotoxic effector cells, was diminished in the lungs of *M. tuberculosis*-infected CD4<sup>-/-</sup> mice. As tuberculosis is frequently associated with HIV infection and a subsequent loss of CD4<sup>+</sup> T cells, understanding the interaction between CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets during the immune response to *M. tuberculosis* is imperative for the design of successful vaccination strategies. *The Journal of Immunology*, 2001, 167: 6991–7000.

The importance of the CD4<sup>+</sup> T cell subset for generation and maintenance of CD8<sup>+</sup> effector cells in vivo has been examined in studies using viral infections or noninfectious Ags. It was proposed that the role of CD4<sup>+</sup> T cells is to condition APCs for the optimal priming of cytotoxic responses via CD40-CD40 ligand (CD40L)<sup>4</sup> interactions (1–3). However, the requirement for APC conditioning by CD4<sup>+</sup> T cells before CD8<sup>+</sup> T cell priming can be overcome by virally infected APCs. Although in some viral infections, such as HSV (4), CD4<sup>+</sup> T cell help is required for the induction of primary CD8<sup>+</sup> T cell-mediated responses, development of CD8<sup>+</sup> T cell effector responses during a number of viral infections appears to be independent of helper cells. Indeed, induction of primary CD8<sup>+</sup> T cells against influenza A virus (5), ectromelia virus (6), and lymphocytic choriomeningitis virus (LCMV) (7, 8) can occur in the absence of CD4<sup>+</sup> T cell help, although the presence of CD4 T cells can augment the response (9). However, CD4<sup>+</sup> T cells have been shown to be important for the maintenance of CD8<sup>+</sup> T cell effector functions and control of chronic viral infections. During LCMV infection, the development of CD8<sup>+</sup> memory T cells and subsequent resistance to challenge is impaired in CD4<sup>+</sup> T cell-deficient mice (10) and a virus carrier state is established in the absence of T cell help (11). Furthermore, CD4<sup>+</sup> T cells are required to sustain CD8<sup>+</sup> cytotoxic

responses (12) and CD8<sup>+</sup> CTL memory responses are exhausted in the absence of CD4<sup>+</sup> helper cells (13). Progressive loss of both cytotoxic and cytokine-secreting functions of CD8<sup>+</sup> T cells during gammaherpesvirus infection in the absence of CD4<sup>+</sup> T cells was also reported (14). In most cases, the mechanisms by which CD4 T cells participate in maintaining CD8<sup>+</sup> T cell responses in viral infections have not been clearly elucidated.

CD8<sup>+</sup> T cells contribute to immune protection against certain intracellular noncytoplasmic pathogens, but the interaction between CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets during bacterial infections has not been investigated. Protective immunity against *Mycobacterium tuberculosis* involves CD4<sup>+</sup> and CD8<sup>+</sup> T cells and mice lacking either or both T cell subsets are more susceptible to infection than the wild-type (WT) mice (15–20). Previously we reported that although IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells emerge in the lungs of infected CD4<sup>-/-</sup> mice, they are not sufficient for protection (15). Chronically infected mice depleted of CD4<sup>+</sup> T cells were unable to prevent reactivation of infection despite normal levels of IFN- $\gamma$  and nitric oxide synthase 2 production, suggesting roles for CD4<sup>+</sup> T cells in addition to cytokine production and macrophage activation (21). In the present study, we examined the effect of CD4<sup>+</sup> T cell deficiency on the development of cytokine production and cytotoxic functions of CD8<sup>+</sup> T cells during acute tuberculosis. Our data indicate that priming and amplification of mycobacteria-specific CD8<sup>+</sup> T cells occurred in the absence of CD4<sup>+</sup> T cells, as did migration of cytokine-secreting CD8<sup>+</sup> T cells to the lungs following *M. tuberculosis* infection. However, development of cytotoxic CD8<sup>+</sup> effector cells in the lungs was substantially diminished in the absence of CD4<sup>+</sup> T cells. These results suggest that CD4<sup>+</sup> T cells contribute to protective immunity not only by secretion of antimicrobial cytokines such as IFN- $\gamma$  but also by providing help for maintaining optimal CD8<sup>+</sup> T cell cytotoxic responses.

## Materials and Methods

### Mice

Eight- to 10-wk-old C57BL/6 (WT; Charles River Breeding Laboratories, Rockland, MA, or The Jackson Laboratory, Bar Harbor, ME), CD4<sup>-/-</sup>, and MHC class II<sup>-/-</sup> mice were used. MHC class II<sup>-/-</sup> (22) and CD4<sup>-/-</sup> (8) breeding pairs were originally obtained from Dr. D. Mathis and Dr. T.

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Received for publication June 19, 2001. Accepted for publication October 16, 2001.

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<sup>1</sup> This work was supported by National Institutes of Health Grant AI37859 and American Cancer Society Junior Faculty Award (to J.L.F.) and Grant T32-CA82084 (N.V.S.).

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<sup>4</sup> Abbreviations used in this paper: CD40L, CD40 ligand; LCMV, lymphocytic choriomeningitis virus; DC, dendritic cell; BrdU, bromodeoxyuridine; WT, wild type.

Mak, respectively, and then from The Jackson Laboratory. These mice were bred in the pathogen-free facility at the University of Pittsburgh School of Medicine (Pittsburgh, PA). All mice were maintained in specific pathogen-free Biosafety Level 3 facilities.

### Bacteria and infections

*M. tuberculosis* (Erdman strain; Trudeau Institute, Saranac Lake, NY) was passaged through mice, grown in culture once, and frozen in aliquots. Before infection, an aliquot was thawed, diluted in PBS containing 0.05% Tween 80, and sonicated for 10 s in a cup horn sonicator. Mice were infected by aerosol with ~50–100 live bacilli as determined by viable counts on 7H10 agar plates (Difco, Detroit, MI);  $10^7$  CFU/ml were placed in a nebulizer, and mice were exposed for 20 min, followed by 5 min of air only, using a nose-only unit (Intox, Albuquerque NM) as previously described (23). For the CTL assay in which cells were cultured before use in lysis assays, mice were injected i.v. via tail vein with  $2 \times 10^5$  live bacilli in 100  $\mu$ l.

### Culture and infection of DCs

Dendritic cells (DCs) were grown from murine bone marrow precursors and cultured for 5 days using methods described previously (24). For DC infection, nonadherent cells were harvested, adjusted to  $0.5 \times 10^6$  cells/ml in DC medium containing recombinant murine GM-CSF, and dispersed into 25-cm<sup>2</sup> culture flasks (Costar, Cambridge, MA) for infection. Cells were infected for 16–18 h at multiplicity of infection of 3–5. Extracellular bacteria were separated from cells by low-speed centrifugation and fresh medium was added to the cells. For FACS analyses, infected and uninfected DCs were cultured in fresh medium for an additional 24 h. The percentage of infection was estimated by staining aliquots of cells by the Kinyoun method for acid-fast bacteria (Difco). Routinely, ~50% of DCs were infected.

### FACS analyses of cell surface markers

Lung and mediastinal lymph node cells were obtained from mice infected for various periods of time as described previously (24). Cells were stained for cell surface markers using Abs against CD8 (anti-CD8 CyChrome Ab, clone 53-6.7), CD4 (anti-CD4 CyChrome Ab, clone H129.19), CD44 (anti-CD44 FITC Ab, clone IM7), CD45RB (anti-CD45RB FITC Ab, clone 16A), CD69 (anti-CD69 FITC Ab, clone H1.2F3), and CD25 (anti-CD25 PE Ab, clone PC61). DCs were stained for surface markers using Abs against MHC class II (anti-I-A<sup>b</sup> FITC Ab, clone AF6-120.1), MHC class I (anti-H2D<sup>b</sup> PE Ab, clone KH95), ICAM-1 (anti-CD54 FITC Ab, clone 3E2), B7.1 (anti-CD80 FITC Ab, clone H1.2F3), and B7.2 (anti-CD86 FITC Ab, clone GL-1). All staining procedures were performed in PBS containing 20% mouse serum, 0.1% BSA, and 0.1% sodium azide (FACS buffer) for 30 min at 4°C. All Abs were used at 0.2  $\mu$ g/ $10^6$  cells and obtained from BD PharMingen (San Diego, CA). Cells were fixed with 4% paraformaldehyde for at least 1 h and analyzed by flow cytometry using CellQuest software (BD Immunocytometry Systems, San Jose, CA). Cells were gated on the lymphocyte or monocyte population by forward and side scatter.

### Intracellular staining

Single cell suspensions of lungs at various times postinfection were prepared and staining for intracellular cytokines was performed as described previously (24). Briefly, cells were either stimulated with anti-CD3 (clone 145-2C11, 0.1  $\mu$ g/ml) and anti-CD28 (clone 37.51, 1  $\mu$ g/ml) Abs (BD PharMingen) or left unstimulated for 5–6 h in the presence of 3  $\mu$ M monensin (Sigma-Aldrich, St. Louis, MO). At the end of the stimulation period, cells were stained for CD4 and CD8, fixed, permeabilized, and stained for intracellular cytokines.

### Measurement of BrdU incorporation in vivo

Mice infected with *M. tuberculosis* for 4 wk were administered 1 mg of bromodeoxyuridine (BrdU) in PBS i.p. (catalog no. B5002; Sigma-Aldrich) 24 h before harvest of lungs. Single cell suspensions of lung tissue were stained with FITC-anti-BrdU Ab or FITC-IgG isotype Ab (catalog no. 36634K; BD PharMingen) according to the manufacturer's instructions. Briefly, cells were stained for CD4 and CD8 cell surface Ags, permeabilized in ice-cold 70% ethanol, washed, and incubated in denaturing solution (2 M HCl, 0.5% BSA) for 20 min at room temperature. Cells were washed and pellet was resuspended in 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, pH 8.5. Cells were incubated for 2 min at room temperature, washed, and stained with FITC-anti-BrdU or FITC-IgG isotype Abs for 30 min. Cells were washed and fixed with 4% paraformaldehyde and analyzed by flow cytometry.

### IFN- $\gamma$ ELISA

DCs uninfected or infected (multiplicity of infection 4) for 24 h as described were plated in 96-well U-bottom plates (Corning Glass, Corning, NY) at  $10^4$  cells/well in DMEM supplemented with 10% certified FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES (Life Technologies, Grand Island, NY), 50  $\mu$ M 2-ME (Sigma-Aldrich), 30  $\mu$ g/ml gentamicin (Life Technologies, Gaithersburg, MD), 15–20 U/ml murine IL-2 (Boehringer Mannheim, Indianapolis, IN), and 1 mM aminoguanidine (Sigma-Aldrich). Lung cells were harvested from mice infected for 4 wk via aerosol, and single cell suspensions were obtained as described (24). Macrophages were depleted by adherence on plastic petri dishes for 2 h at 37°C. Lung cells were added at  $10^5$  cells/well and cultured with APC for 3 days. Culture of cells in medium alone served as a baseline. As a positive control, cells were stimulated with Con A (Boehringer Mannheim) at 5  $\mu$ g/ml. Supernatants were harvested after 3 days of culture and IFN- $\gamma$  production was measured by sandwich ELISA using Abs R4-A62 and XMG1.2 (biotinylated) (BD PharMingen), according to the manufacturer's protocol. Recombinant murine IFN- $\gamma$  used to generate a standard curve was a gift from Genentech (San Francisco, CA).

### Culture of lung and lymph node cells

Lung and lymph node cells from mice uninfected or infected for 2–5 wk were obtained as described above and plated in 96-well U-bottom plates (Corning Glass) in DMEM supplemented with 10% certified FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES (Life Technologies), 50  $\mu$ M 2-ME (Sigma-Aldrich), 30  $\mu$ g/ml gentamicin (Life Technologies), 15–20 U/ml murine IL-2 (Boehringer Mannheim), and 1 mM aminoguanidine (Sigma-Aldrich) at  $2 \times 10^5$  cells/well. MHC class II<sup>-/-</sup> DCs infected for 18–24 h as described above were added to the cell cultures at  $6.5\text{--}7 \times 10^3$  viable cells/well. After 2–3 days of culture, 100  $\mu$ l of medium were removed from each well and replaced with fresh medium containing IL-2. Cells were cultured for an additional 3–4 days before FACS analyses and CTL assays.

### Cytotoxicity assays

Lymphocytes harvested from 5- to 7-day stimulation cultures were tested in a 4-h <sup>51</sup>Cr release assay as previously described (23). Macrophages were infected for 48 h or left uninfected, labeled with 100  $\mu$ l of Na<sup>51</sup>CrO<sub>4</sub> (Amersham, Arlington Heights, IL) and used as targets. Target cells were added to wells of 96-well U-bottom plates (Corning) at  $4 \times 10^3$  cells/well and allowed to adhere for 20 min before addition of T cells. Cultured cells were added at various E:T ratios in a total volume of 0.1 ml in DMEM supplemented with 10% certified FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, and 50  $\mu$ M 2-ME, and assay was conducted for 4 h. After 4 h, 85  $\mu$ l of supernatant was removed from each well without disturbing the cells and counted in a gamma counter. Spontaneous release was determined by culturing target cells in medium alone, and total release was determined by adding 0.1% Triton X-100 to target cells. Percent of specific lysis was calculated by the formula:  $100 \times (\text{experimental counts per minute} - \text{spontaneous counts per minute} / \text{total counts per minute} - \text{spontaneous counts per minute})$ .

### Ab-dependent redirected target lysis assay

Lung cells harvested from WT mice treated with anti-CD4 Ab (GK1.5, 0.5 mg/injection) i.p. 6 days before CTL assay or CD4<sup>-/-</sup> mice were directly used as effectors in a 4-h <sup>51</sup>Cr release assay. FcR<sup>+</sup> P815 (H-2<sup>d</sup>) murine mastocytoma tumor cells were labeled with 100  $\mu$ l of Na<sup>51</sup>CrO<sub>4</sub> (Amersham) for 1 h, washed extensively, and added to wells of 96-well U-bottom plates (Corning) at  $4 \times 10^3$  cells/well. Lung cells were added at various E:T ratios and the assay was conducted in the presence or absence of anti-TCR $\alpha\beta$  mAb (clone H57-597, used at  $10^{-4}$   $\mu$ g/ml; BD PharMingen) for 4 h. Percentage of specific lysis was calculated as described above.

### Ribonuclease protection assay

Mice were infected via aerosol with ~100 viable bacilli, and lungs were harvested at various times postinfection and flash-frozen. Total RNA was isolated from frozen tissues using TRIzol (Life Technologies) according to the manufacturer's instructions, with an additional phenol-chloroform extraction after the TRIzol extraction. Expression of cytokine genes was examined using the RiboQuant ribonuclease protection assay system (BD PharMingen) using mCK-1 ribonuclease protection assay template set. Intensities of the bands on the autoradiographs were quantitated by densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA). The results are expressed as the ratio of band intensities of genes of interest and housekeeping gene, GAPDH or L32.

### Statistical analysis

Data were analyzed by comparison of WT and CD4<sup>-/-</sup> mice at each time point, with 3–4 mice per time point (see text and figures); unpaired *t* tests were used. Prism 2.0 (GraphPad, San Diego, CA) was used for statistical analysis. Values of *p* < 0.05 were considered significant, and *p* values are not shown for nonsignificant differences. Each experiment was repeated at least once to ensure reproducibility (see text and figures).

## Results

### Priming of mycobacteria-specific CD8<sup>+</sup> T cells in vivo and in vitro does not require CD4<sup>+</sup> T cell help

To address the requirement for CD4<sup>+</sup> T cell help in priming mycobacteria-specific CD8<sup>+</sup> T cells in vitro, we used DCs from MHC class II<sup>-/-</sup> mice; the absence of MHC class II blocks their ability to specifically stimulate CD4<sup>+</sup> T cell responses. It is believed that CD4<sup>+</sup> T cells function to condition APCs via CD40-CD40L interactions for improved T cell priming interactions (1–3). Substantial increases in cell surface expression of MHC class I, B7.1, and B7.2 were observed when MHC class II<sup>-/-</sup> DCs were infected with *M. tuberculosis* for 64–66 h (Fig. 1A), suggesting that, as demonstrated previously by our group and others (25–27), infection with *M. tuberculosis* was by itself sufficient to induce phenotypic maturation of DCs in the absence of CD4<sup>+</sup> T cells. Expression of MHC class II molecules on the surface of these DCs was not detectable (data not shown).

The ability of *M. tuberculosis*-infected MHC class II<sup>-/-</sup> DCs to prime responses of naive CD8<sup>+</sup> T lymphocytes was evaluated. Lymph nodes from naive CD4<sup>-/-</sup> mice were cultured with infected MHC class II<sup>-/-</sup> DCs in the presence of IL-2 for 5 days. At the end of culture period, the resultant CD8<sup>+</sup> T cell population (~98% CD8<sup>+</sup>, data not shown) was tested for cytotoxic activity against uninfected or *M. tuberculosis*-infected macrophages. Primed CD8<sup>+</sup> T cells lysed infected targets, but not uninfected targets (Fig. 1B). Therefore, priming, expansion, and acquisition of cytotoxic function by mycobacteria-specific CD8<sup>+</sup> T cells can occur in the absence of CD4<sup>+</sup> T cells in vitro. However, IL-2 was supplied to these in vitro cultures during priming, which would bypass an important function of CD4<sup>+</sup> T cells.

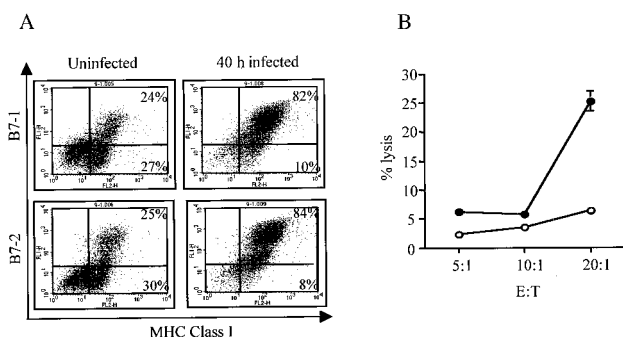
To address in vivo priming of *M. tuberculosis*-specific CD8<sup>+</sup> T cells in a CD4<sup>+</sup> T cell-deficient environment, the activation pro-

files of CD8<sup>+</sup> T lymphocytes in the mediastinal lymph nodes of aerogenically infected CD4<sup>-/-</sup> and WT mice were compared. CD8<sup>+</sup> T cells in the lymph nodes of uninfected mice express very low levels of the activation markers CD69 and CD25. Following infection, the numbers of CD69<sup>+</sup> and CD25<sup>+</sup>CD8<sup>+</sup> T cells in the lymph node increase (24). Numbers and activation profiles of CD8<sup>+</sup> T cells in the lymph nodes of WT and CD4<sup>-/-</sup> mice were comparable after aerogenic infection with *M. tuberculosis* (Fig. 2, A and B). The patterns of CD69 and CD25 expression in the lymph nodes from WT and CD4<sup>-/-</sup> T cell-deficient mice were comparable (Fig. 2, A and B). During the first 2 wk of infection, higher percentages of CD8<sup>+</sup> T cells expressed CD69 and CD25 in the lymph nodes of the knockout mice. Throughout the course of infection, lymph node CD8<sup>+</sup> T cells were CD44<sup>low</sup>CD45RB<sup>high</sup> in both mouse strains; the expression of these markers was similar in the lymph nodes from infected and naive mice (data not shown).

To examine whether CD8<sup>+</sup> T cells undergoing priming in the lymph nodes of CD4<sup>+</sup> T cell-deficient mice could develop into cytotoxic effectors, lymph node cells from 4-wk-infected CD4<sup>-/-</sup> mice were restimulated with infected MHC class II<sup>-/-</sup> DCs for 5–7 days in the presence of rIL-2. CD8<sup>+</sup> T cell-enriched populations from lymph nodes of both WT and CD4<sup>-/-</sup> mice were equally capable of lysing *M. tuberculosis*-infected macrophages (Fig. 2B). CD8<sup>+</sup> T cells from the lymph nodes of MHC class II<sup>-/-</sup> mice yielded similar results (data not shown). There were 6- to 7-fold more CD8<sup>+</sup> T cells in the lymph nodes of 4-wk-infected WT and CD4<sup>-/-</sup> mice as compared with naive mice, suggesting an increased numbers of CTL precursors in the lymph nodes of both groups of mice following infection. Our experimental conditions did not allow differentiation between in vitro priming during the 5- to 7-day culture period and restimulation of cells already primed in vivo. However, the data demonstrate that lymph nodes of CD4-deficient mice contained CD8<sup>+</sup> T cells fully capable of developing into cytotoxic effector cells.

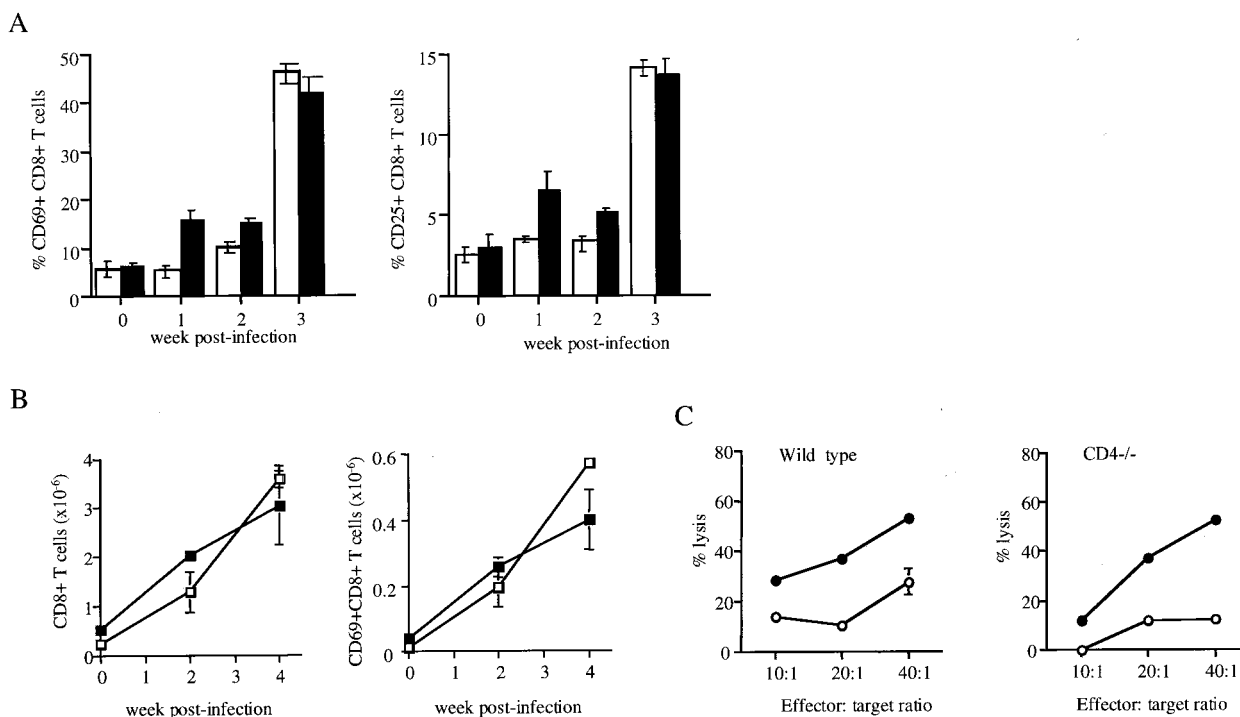
### Acquisition of activated phenotype by CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cell help

CD4<sup>+</sup> T cells may be important for optimal activation of effector CD8<sup>+</sup> T cells, as suggested in certain viral experimental systems. We previously reported that migration of CD8<sup>+</sup> T cells into the lungs following i.v. infection was not diminished in the absence of CD4<sup>+</sup> T cells; in fact, percentages of CD8<sup>+</sup> T cells in the lungs of CD4<sup>+</sup> T cell-deficient mice were ~2-fold higher than in the lungs of control mice up to 6 wk postinfection (Fig. 3B). It was not clear whether the increase in CD8<sup>+</sup> T cells observed in the lungs of infected mice was due to the continuous migration of newly primed CD8<sup>+</sup> T cells into the lungs or to the proliferation of CD8<sup>+</sup> T cells at this site. Because CD8<sup>+</sup> T cells produce significantly less IL-2 than CD4<sup>+</sup> T cells, the absence of CD4<sup>+</sup> T cells might negatively affect proliferation of CD8<sup>+</sup> effectors in the lungs. To address this issue, 4-wk-infected WT and CD4<sup>-/-</sup> mice were administered BrdU, and 24 h later the lungs were harvested. Incorporation of BrdU into T cells was assessed by flow cytometry. In the lungs of WT mice, 30–40% of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were BrdU positive (data not shown). Similar percentages of BrdU-positive CD8<sup>+</sup> T cells were observed in the lungs of WT and CD4<sup>-/-</sup> mice (Fig. 3C), suggesting that absence of CD4<sup>+</sup> T cells did not affect the proliferation of CD8<sup>+</sup> T cells in *M. tuberculosis*-infected mice.



**FIGURE 1.** In vitro priming of cytotoxic CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cell help. *A*, Bone marrow-derived DC were infected with *M. tuberculosis* or left uninfected for 40 h, stained for MHC class I, B7.1, and B7.2, and analyzed by flow cytometry. Cells were gated on monocyte population by size, and expression of markers within monocyte gate was analyzed. Shown is a representative experiment that was repeated twice. *B*, Lymph node cells from three CD4<sup>-/-</sup> naive mice were pooled and cultured for 5 days with *M. tuberculosis*-infected MHC class II<sup>-/-</sup> DC and used as effectors in a 4-h <sup>51</sup>Cr release cytotoxicity assay. Targets: uninfected bone marrow-derived macrophages (○) and macrophages infected for 42 h with *M. tuberculosis* (●). Error bars represent SE.





**FIGURE 2.** Priming of CD8<sup>+</sup> T cells in the lymph nodes of *M. tuberculosis*-infected mice in the absence of CD4<sup>+</sup> T cells. **A**, WT (open bars) and MHC class II<sup>-/-</sup> (filled bars) mice were infected with *M. tuberculosis* bacilli via aerosol, and lung-draining lymph nodes were harvested 0, 1, 2, 3, and 6 wk postinfection. Cells were stained for CD8, CD69, and CD25, gated on lymphocyte population by size, and analyzed by flow cytometry. Cells were further gated on CD8, and CD69<sup>+</sup> and CD25 expression in the gate was analyzed. Each bar corresponds to three to six mice. Similar data were obtained using lymph nodes from CD4<sup>-/-</sup> mice. **B**, WT (□) and CD4<sup>-/-</sup> (■) mice were infected with *M. tuberculosis* bacilli via aerosol, and lung-draining lymph nodes were harvested 0, 1, 2, 3, and 4 wk postinfection. Numbers of viable cells in lymph nodes were counted by trypan blue exclusion. Cells were stained for CD8 and CD69, gated on lymphocyte population by size, and analyzed by flow cytometry. The total numbers of CD8<sup>+</sup> T cells were calculated based on percentages of the CD8<sup>+</sup> and CD8<sup>+</sup>CD69<sup>+</sup> cells in the lymphocyte gate. **C**, Lymph node cells from WT and CD4<sup>-/-</sup> mice infected for 4 wk were cultured for 5 days with *M. tuberculosis*-infected MHC class II<sup>-/-</sup> DC and used in a 4-h <sup>51</sup>Cr release cytotoxicity assay. Targets: uninfected bone marrow-derived macrophages (○) and macrophages infected for 42 h (●). Error bars represent SE; at some time points, error bars are too small to be seen on the graph.

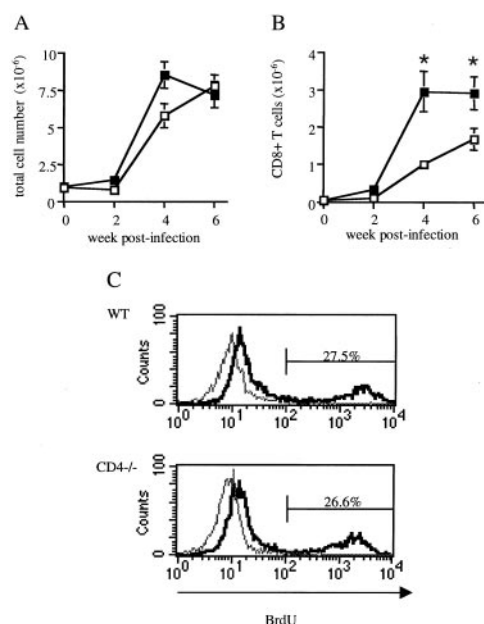
The kinetics of acquisition of a CD44<sup>high</sup> phenotype by CD8<sup>+</sup> T cells from WT and CD4<sup>-/-</sup> mice were similar, although the expression peaked slightly earlier (wk 2) in CD4<sup>-/-</sup> mice (Fig. 4A). The up-regulation of CD44 expression by CD8<sup>+</sup> T cells was concomitant with a decrease in CD45RB expression in both WT and CD4<sup>-/-</sup> mice, suggesting an effector/memory phenotype (Fig. 4A). In the lungs of both WT and CD4<sup>-/-</sup> mice, CD69<sup>+</sup>CD8<sup>+</sup> T cells were detected as early as 1 wk postinfection and continued to increase throughout the course of infection (Fig. 4, B and C). The activation profiles of CD8<sup>+</sup> T cells from MHC class II<sup>-/-</sup> mice were comparable to those of CD8<sup>+</sup> T cells from CD4<sup>-/-</sup> and WT mice (data not shown). These data indicate that absence of CD4<sup>+</sup> T cells had no obvious effect on recruitment of primed CD8<sup>+</sup> T cells to the lungs and subsequent CD8<sup>+</sup> T cell activation during infection.

#### Cytokine production by CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cells

Previously, we demonstrated that IFN- $\gamma$  production in the lungs of mice devoid of CD4<sup>+</sup> T cells was reduced by >50% as compared with that in WT mice during the first 2 wk after i.v. infection (15), indicating that early IFN- $\gamma$  production is primarily by CD4<sup>+</sup> T cells. We further examined the effect of a CD4<sup>+</sup> T cell deficiency on the development of cytokine-secreting CD8<sup>+</sup> T cells during acute *M. tuberculosis* infection. In the lungs of aerogenically infected mice, cytokine-producing T cells emerged by 2 wk postinfection, and the percentages of IFN- $\gamma$ -secreting cells reached a plateau by wk 4 postinfection (data not shown). Cytokine produc-

tion by CD8<sup>+</sup> T cells from the lungs of WT and CD4<sup>-/-</sup> mice at 4 and 6 wk post-aerosol infection was assessed by intracellular cytokine staining. In these experiments, brief (5–6 h) stimulation of lung cells with anti-CD3 and anti-CD28 Abs followed by intracellular cytokine staining allowed us to examine the total cytokine producing potential of the T cells. Stimulation with anti-CD3/anti-CD28 Ab induced IFN- $\gamma$  and TNF- $\alpha$  secretion by CD8<sup>+</sup> T cells from both WT and CD4<sup>-/-</sup> mice, and the percentage of CD8<sup>+</sup> T cells primed for cytokine production was similar in both WT and CD4<sup>-/-</sup> mice (Fig. 5, A and B).

To confirm that cytokine-producing cells were Ag specific, lung cells from 4-wk-infected WT and CD4<sup>-/-</sup> mice were cultured with uninfected or *M. tuberculosis*-infected DCs for 72 h and culture supernatants were assayed for IFN- $\gamma$  by ELISA. WT mice were depleted of CD4<sup>+</sup> T cells 4 days before the lung harvest by administration of GK1.5 Ab i.p. (resulting in 95% depletion, data not shown) so that similar populations of lung cells could be compared. Low levels of IFN- $\gamma$  were produced when cells were cultured with uninfected DCs or in the medium alone (Fig. 5C). In contrast, stimulation with infected DCs induced robust IFN- $\gamma$  secretion by CD8<sup>+</sup> T cells comparable to that induced by Con A (Fig. 5D), indicating that lungs of both WT and CD4<sup>-/-</sup> mice contained *M. tuberculosis*-specific cytokine-producing CD8<sup>+</sup> T cells. Specific IFN- $\gamma$  production was also assessed by intracellular cytokine staining after short-term (12 h) culture of lung CD8<sup>+</sup> T cells and infected DCs; under these conditions, similar percentages of CD8<sup>+</sup> T cells from WT and CD4<sup>-/-</sup> mice produced IFN- $\gamma$  (data not shown). We also examined ex vivo cytokine secretion by



**FIGURE 3.** CD8<sup>+</sup> T cell infiltration of the lungs following *M. tuberculosis* infection in WT and CD4<sup>-/-</sup> mice. WT (□) and CD4<sup>-/-</sup> (■) mice were infected with *M. tuberculosis* bacilli via aerosol and lung cells were harvested 0, 2, 4, and 6 wk postinfection. **A**, Numbers of viable cells in lungs were counted by trypan blue exclusion. For all time points,  $p > 0.05$ . **B**, Cells were stained for CD8, gated on lymphocyte population by size, and analyzed by flow cytometry. The total numbers of CD8<sup>+</sup> T cells were calculated based on percentages of the CD8<sup>+</sup> in the lymphocyte gate. \*,  $p < 0.01$ . **C**, WT and CD4<sup>-/-</sup> mice infected with *M. tuberculosis* for 4 wk were administered BrdU i.p. and lungs were harvested 24 h later. Cells were stained for CD8, permeabilized, stained with anti-BrdU Ab (solid line) or isotype control (gray line), and analyzed by flow cytometry, gating on the CD8<sup>+</sup> cells. Shown is a representative experiment, and the experiment was repeated two times.

CD8<sup>+</sup> T cells in the absence of exogenous TCR stimulation, a measure of T cells secreting cytokines in vivo at the site of infection immediately before harvest. We observed that equal percentages of unstimulated CD8<sup>+</sup> T cells from lungs of CD4<sup>-/-</sup> or WT mice secreted IFN- $\gamma$  (Fig. 5D). Similar data were obtained using MHC class II<sup>-/-</sup> mice (data not shown). Previously we have demonstrated that under conditions described above, IFN- $\gamma$  cannot be detected in lung T cells from uninfected mice (24), suggesting that levels of nonspecific staining are minimal in our assays. These data indicate that development of cytokine-producing CD8<sup>+</sup> T cells during *M. tuberculosis* infection does not require CD4<sup>+</sup> T cells.

#### Cytotoxic activity of lung CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cell help

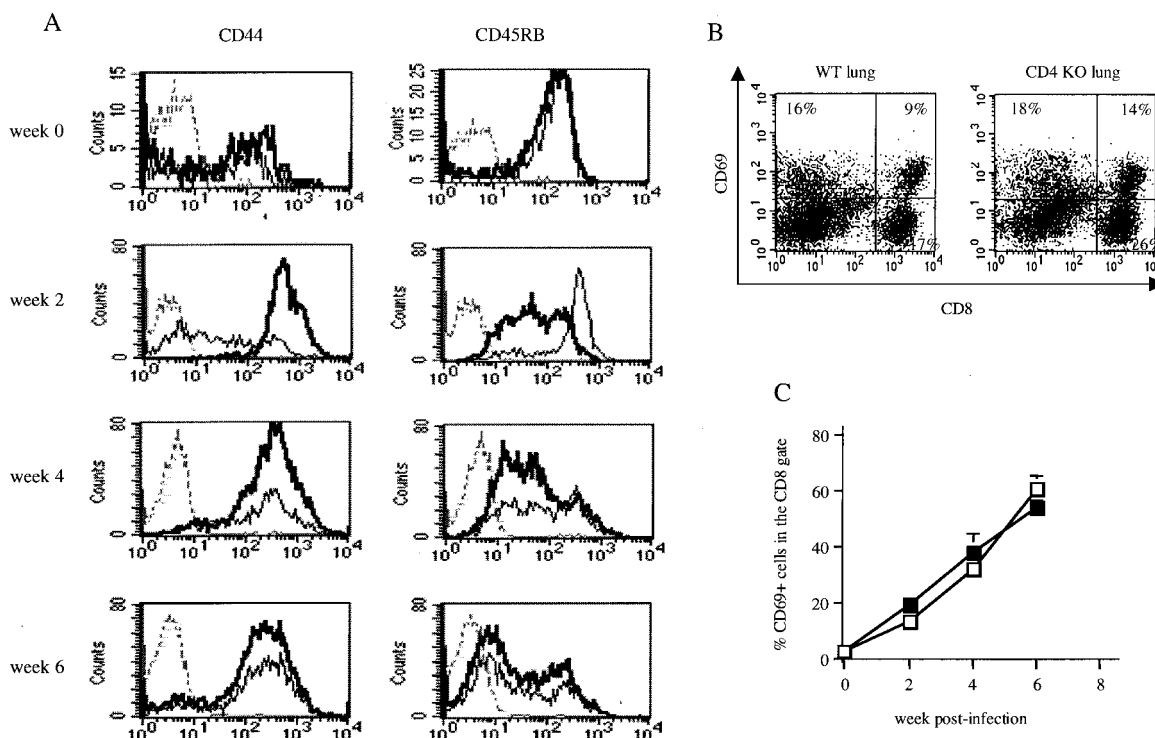
The progressive loss of cytotoxic function of CD8<sup>+</sup> T cells during viral infections in the absence of CD4<sup>+</sup> T cells has been described. Previously, we demonstrated that CD8<sup>+</sup> T cells from the lungs of *M. tuberculosis*-infected mice expressed perforin in vivo and functioned as CTL after in vitro restimulation (23). The in vitro cytotoxicity mediated by cultured CD8<sup>+</sup> T cells from lungs of infected mice depended almost exclusively on perforin (23). We investigated whether the absence of CD4<sup>+</sup> T cells affected the cytotoxic potential of mycobacteria-specific CD8<sup>+</sup> T cells in the infected lungs. Lung cells harvested from either WT or CD4<sup>-/-</sup> mice infected for 3–6 wk were cultured with *M. tuberculosis*-infected MHC class II<sup>-/-</sup> DCs for 5–7 days in the presence of IL-2. At the end of the culture period, the cytotoxic activity of the CD8-enriched populations was tested in a 4-h <sup>51</sup>Cr release assay using *M.*

*tuberculosis*-infected macrophages as targets. In several independent experiments, we observed substantial reductions in the specific lysis mediated by CTLs from the lungs of CD4<sup>-/-</sup> and MHC class II<sup>-/-</sup> mice (Fig. 6A and data not shown), suggesting that the cytotoxic activity of *M. tuberculosis*-specific CTL was compromised in the absence of CD4<sup>+</sup> T cells.

In vitro culture before testing CD8<sup>+</sup> T cells in a CTL assay can introduce variables, making comparison of the cytotoxic potential of the lung T cells from CD4<sup>-/-</sup> and WT mice less clear. To directly assess the effects of CD4<sup>+</sup> T cells on the cytotoxic potential of CD8<sup>+</sup> T cells in the lungs of the *M. tuberculosis*-infected mice, Ab-redirected target lysis assays were performed. Lung cells from WT and CD4<sup>-/-</sup> mice infected for 4 wk were used as effector T cells. WT mice were depleted of CD4<sup>+</sup> T cells by administration of anti-CD4 Ab i.p. 4 days before the cell harvest so that both populations would be comparable in terms of T cell population; this short-term CD4 T cell depletion has no effect on *M. tuberculosis* infection (data not shown). The lungs of both groups of mice at the time of harvest were comprised of ~60% CD8<sup>+</sup> T cells and 20% CD4<sup>-</sup>CD8<sup>-</sup> cells, and contained no CD4<sup>+</sup> T cells (data not shown). The mean fluorescence intensity of TCR $\alpha\beta$  staining in the lungs of WT and CD4<sup>-/-</sup> mice was 106 and 90, respectively (data not shown). The cytotoxic activity of freshly harvested lung cells from both WT and knockout mice was tested in the 4-h <sup>51</sup>Cr release assay against FcR<sup>+</sup> P815 target cells in the presence or absence of anti-TCR $\alpha\beta$  Ab. The Ab binding to the T cells triggers the release of cytotoxic granules, and the Fc portion of the Ab binds to the FcR on the target cells, bringing the T cells into close proximity with the P815 cells. Only those cells primed to be cytotoxic will lyse the targets in this assay. As shown in Fig. 6B, lung cells from WT mice readily lysed target cells in the presence of anti-TCR Ab. That no more than 30% of specific lysis was achieved in our assays was not surprising, because only a subset of lymphocytes in the lungs of *M. tuberculosis*-infected mice express perforin detectable by immunohistochemistry in vivo (23). In contrast, T cells from the lungs of CD4<sup>-/-</sup> mice failed to lyse target cells in the presence of redirecting Ab (Fig. 6B), confirming that the cytotoxic activity of CD8<sup>+</sup> T cells was compromised in the absence of CD4<sup>+</sup> T cells. In a separate ongoing study, we also observe that the frequency of cytotoxic precursors (as measured by limiting dilution assay) in the lungs of CD4<sup>-/-</sup> mice is reduced severalfold as compared with that of WT CD8<sup>+</sup> T cells (data not shown).

#### Production of IL-2 and IL-15 in the lungs of CD4-deficient mice

The above results suggested a defect in the development of cytotoxic effectors in CD4<sup>-/-</sup> mice. IL-2 and IL-15 are involved in development of cytotoxic functions of CD8<sup>+</sup> T cells, NK cells, and lymphokine-activated killer cells (28–32). As CD4<sup>+</sup> T cells are the major source of IL-2, the cytotoxic activity of CD8<sup>+</sup> T cells might crucially depend on the production of IL-2 by this cell subset. In addition, CD4<sup>+</sup> T cells may increase IL-15 secretion by macrophages and DCs. WT and CD4<sup>-/-</sup> mice were infected via aerosol, lungs were harvested 0, 3, and 4 wk postinfection, and cytokine expression was measured using RNase protection assays. Cytokine expression in the lungs of uninfected mice was virtually undetectable (Fig. 7). At 3 and 4 wk postinfection, IL-2 levels were 2- and 4-fold lower, respectively, in CD4<sup>-/-</sup> mice compared with WT mice (Fig. 7A). IL-15 mRNA levels also were reduced in the CD4<sup>-/-</sup> lungs at 3 and 4 wk postinfection, compared with WT mice (Fig. 7B). In a separate experiment, IL-2 and IL-15 were ~6- and ~2-fold lower, respectively, in CD4<sup>-/-</sup> mice compared with WT mice at 2 wk postinfection, with differences at 4 wk similar to the previous experiment (data not shown); in that experiment, a



**FIGURE 4.** Changes in activation profiles of CD8<sup>+</sup> T cells in the lungs of WT and CD4<sup>-/-</sup> mice during *M. tuberculosis* infection. Mice were infected via aerosol, and lung cells were harvested 0, 2, 4, and 6 wk postinfection, stained for CD8, CD44, CD45RB, and CD69, gated on lymphocyte population by size, and analyzed by flow cytometry. **A**, Cells from WT (thin solid lines) or CD4<sup>-/-</sup> (thick solid lines) lungs were further gated on CD8, and expression of CD44 and CD45RB within the gate was analyzed. Broken lines represent staining with isotype control. Shown is a representative mouse for each time point. Experiment was repeated at least three times. **B**, Expression of CD8 and CD69 in the lungs 1 wk postinfection. Shown is a representative mouse, and the experiment was repeated three times. **C**, Cells were harvested from lungs of WT (□) and CD4<sup>-/-</sup> (■) mice, stained for CD8 and CD69, and gated on lymphocytes. Cells were further gated on CD8, and expression of CD69 in the gate was analyzed. Each time point represents three to six mice. Error bars represent SE.

3-wk time point was not obtained. We also observed that levels of IL-10 were higher in the WT mice (Fig. 7C). One possible explanation for this observation is that CD4<sup>+</sup> T cells produce IL-10 during the infection. However, we have not been able to detect IL-10 production by CD4<sup>+</sup> T cells stimulated ex vivo (our unpublished data). Alternatively, CD4<sup>+</sup> T cell interaction with macrophages might lead to the production of IL-10 by macrophages.

## Discussion

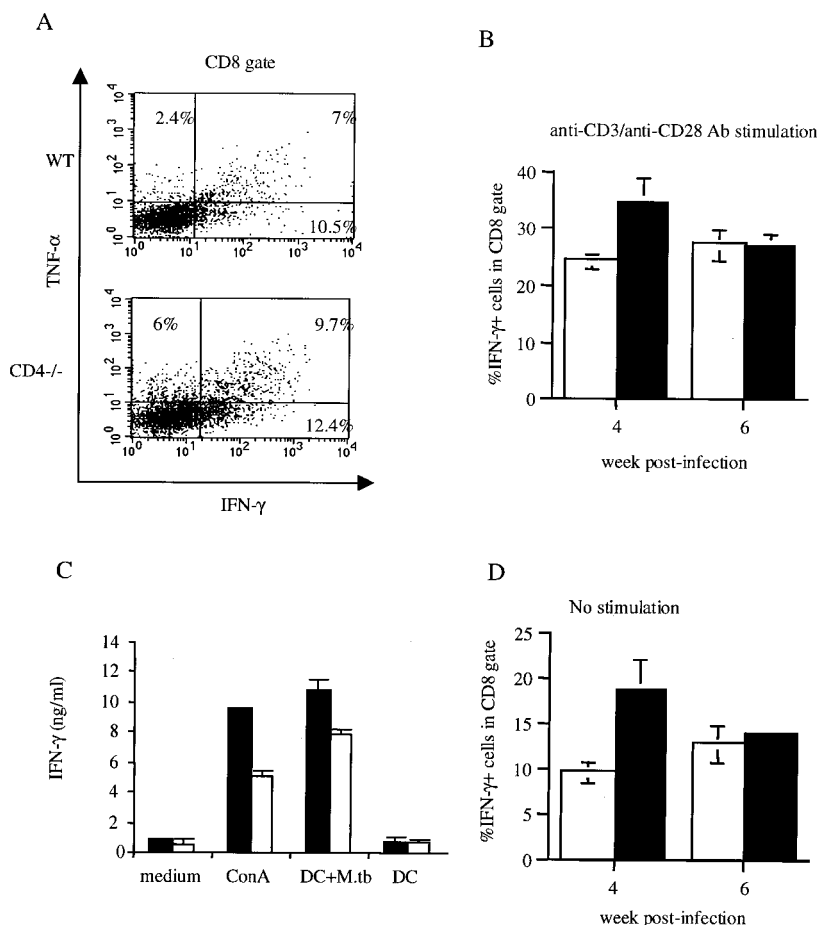
Control of *M. tuberculosis* infection is critically dependent on the presence of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and cytokines such as IFN- $\gamma$  and TNF- $\alpha$  (reviewed in Ref. 33). It is thought that the major function of CD4<sup>+</sup> T cells is production of cytokines. Although the contribution of CD8<sup>+</sup> T cells to control of tuberculosis remains controversial (20, 33), these cells are capable of secreting cytokines (24, 34–36) as well as exerting cytotoxic activity (23, 37) during *M. tuberculosis* infection and following rechallenge (38). Previously, we demonstrated that immune responses during *M. tuberculosis* infection in mice lacking CD4<sup>+</sup> T cells are severely compromised despite the increased numbers of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells (15). Also, chronically infected mice depleted of CD4<sup>+</sup> T cells succumb rapidly to tuberculosis, although overall IFN- $\gamma$  production and inducible nitric oxide synthase production by macrophages in the lungs remain unchanged (21). These results suggested that IFN- $\gamma$  production by CD4<sup>+</sup> T cells is not the only mechanism by which these cells contribute to control of *M. tuberculosis* infection.

In some systems, the presence of regulatory CD4<sup>+</sup> T cells is required for the optimal induction and maintenance of effector re-

sponses of CD8<sup>+</sup> T cells (reviewed in Refs. 39 and 40). In this study, we examined the effects of CD4<sup>+</sup> T cell deficiency on the development of cytotoxic and cytokine-producing effector functions of CD8<sup>+</sup> T cells during *M. tuberculosis* infection. Priming and migration of CD8<sup>+</sup> T cells to the lungs appeared normal in the absence of CD4<sup>+</sup> T cells. However, the cytotoxic ability, but not cytokine production, of CD8<sup>+</sup> T cells was significantly impaired in CD4<sup>-/-</sup> mice. A reduction in the levels of IL-2 and IL-15 gene expression in the lungs, cytokines essential for the development of cell-mediated cytotoxicity, may play a role in the deficient CTL activity. These results suggest that during *M. tuberculosis* infection an additional function of CD4<sup>+</sup> T cells may be to optimize or maintain CD8<sup>+</sup> T cell cytotoxic responses, possibly through regulation of the cytokine microenvironment.

In most viral systems studied, the mechanisms by which CD4<sup>+</sup> T cells regulate CD8<sup>+</sup> T cell responses and function remain unclear. Development and maturation of precursor CD8<sup>+</sup> T cells into cytotoxic and cytokine-producing effectors is a complex process involving a number of regulatory steps. Activation of CD8<sup>+</sup> T cell effector functions via MHC class I-TCR interactions depends on costimulation and the presence of a variety of cytokines (41, 42). CD4<sup>+</sup> T cells contribute to priming of CD8 T cells by “conditioning” the APC via CD40-CD40L interaction for optimal presentation of Ag to CD8<sup>+</sup> T cells (1–3, 43). Indeed, CD40L has been shown to be pivotal for control of some (44–46) but not all (47) viral infections. The CD4<sup>+</sup> T cell-mediated “conditioning” is thought to involve enhancement of costimulatory function of APCs (48, 49) as well as induction of IL-12 secretion by APCs, which induces IFN- $\gamma$  production by T cells (49–53). In this and

**FIGURE 5.** Cytokine production by lung cells from aerogenically infected WT and CD4<sup>-/-</sup> mice. Lung cells from mice at 4 and 6 wk postinfection were stimulated with anti-CD3 and anti-CD28 Abs in the presence of monensin for 5–6 h. Cells were gated on CD8, and expression of TNF- $\alpha$  and IFN- $\gamma$  within gate was analyzed. **A**, Production of TNF- $\alpha$  and IFN- $\gamma$  by CD8<sup>+</sup> T cells from lungs of infected mouse after anti-CD3/anti-CD28 Ab stimulation. A representative mouse infected for 4 wk is shown, and the experiment was repeated at least two times with two mice per time point. **B**, IFN- $\gamma$  production by anti-CD3/anti-CD28 Ab-stimulated CD8<sup>+</sup> T cells from lungs of WT (open bars) and CD4<sup>-/-</sup> (filled bars) mice. Each time point represents three to six mice. **C**, Cells from the lungs of WT (open bars) and CD4<sup>-/-</sup> (filled bars) mice infected for 4 wk were cultured in the medium alone or with various stimulators for 3 days and supernatants were assayed for the presence of IFN- $\gamma$  by ELISA. CD4<sup>+</sup> T cells were depleted from WT mice 4 days before harvest of cells, and MHC class II<sup>-/-</sup> DCs were used as stimulators so that CD8<sup>+</sup> T cell IFN- $\gamma$  production could be measured. **D**, Ex vivo IFN- $\gamma$  production by unstimulated CD8<sup>+</sup> T cells from lungs of WT (open bars) and CD4<sup>-/-</sup> (filled bars) mice after 5 h of culture in the presence of monensin. Each time point represents three to six mice. Error bars represent SE.



previous studies (25, 27), we demonstrated that infection with *M. tuberculosis* induced maturation of DCs in the absence of T cells, apparently bypassing the need for interaction of the DCs with T cells, and that infected DCs produce IL-12. DCs have been reported to acquire CD8<sup>+</sup> T cell priming capacity after treatment with bacterial products such as LPS (43). Mycobacterial lipopeptides were shown to induce maturation of DCs via Toll-like receptor, suggesting at least one mechanism by which activation of APCs can occur in the absence of CD4<sup>+</sup> T cell help (54). In a recent study, DCs (CD11c<sup>+</sup>) isolated from murine lungs matured and acquired Ag presenting ability upon ex vivo infection with *M. tuberculosis* in the absence of T cells (55). In accordance with the observed effect of *M. tuberculosis* infection on the maturation of MHC class II<sup>-/-</sup> DCs, the infected APCs primed naive CD8<sup>+</sup> T cells in vitro, suggesting that priming of precursor CD8<sup>+</sup> T cells to mycobacterial Ags can occur independently of CD4<sup>+</sup> T cells. The in vitro primed CD8<sup>+</sup> T cells recognized and lysed *M. tuberculosis*-infected macrophages, supporting that the presence of "conditioning" CD4<sup>+</sup> T cells was not required for generation of mycobacteria-specific CTLs. Our data are in agreement with a report demonstrating that mice deficient in CD40L are fully capable of mounting a protective immune response against *M. tuberculosis* delivered i.v. (56). However, CD8<sup>+</sup> T cell function was not addressed in these mice, and it remains to be determined whether CD40L<sup>-/-</sup> mice are capable of controlling a chronic *M. tuberculosis* infection.

The conditions of in vitro assays might not exactly reflect the in vivo environment in which priming of precursor CD8<sup>+</sup> T cells occurs. For example, the APCs generated in vitro by culturing with recombinant cytokines might not be at the same stage of activation

and maturation as APCs present in tissues in vivo. In addition, IL-2, which is primarily produced by CD4<sup>+</sup> T cells, was added to the in vitro cultures but is reduced in vivo in the absence of CD4<sup>+</sup> T cells. Therefore, we examined the priming of CD8<sup>+</sup> T cells in the lung-draining lymph nodes of *M. tuberculosis*-infected mice. Increased numbers of CD8<sup>+</sup> T cells expressing CD69, an early activation marker, in the lymph nodes of both WT and CD4<sup>-/-</sup> mice were observed as early as 1 wk postinfection, suggesting that CD8<sup>+</sup> T cell priming in the lymph nodes was independent of CD4<sup>+</sup> T cells in vivo.

Several reports suggested that CD4<sup>+</sup> T cells are required not for priming but rather for survival and maintenance of effector CD8<sup>+</sup> T cells (reviewed in Ref. 40). In the absence of CD4<sup>+</sup> T cell help, CD8<sup>+</sup> CTL were shown to be activated only transiently, and these cells lost function during the chronic phase of viral infection (14, 57). Exhaustion of CD8<sup>+</sup> T cell responses in chronic viral infection was reported under conditions of CD4<sup>+</sup> T cell deficiency (10, 12), supporting the idea that absence of CD4<sup>+</sup> T cells may not only prevent the expansion of CD8<sup>+</sup> T cells but also induce their elimination. In a gammaherpesvirus model, stimulation through CD40 reduced reactivation of a latent infection in CD4<sup>+</sup> T cell-deficient mice, although augmented CD8<sup>+</sup> T cell function could not be demonstrated (58). In our experiments, infiltration of activated (CD44<sup>high</sup>CD45<sup>low</sup>) CD8<sup>+</sup> T cells into the lungs was observed throughout the course of infection in WT and CD4<sup>+</sup> T cell-deficient mice. Substantial numbers of CD8<sup>+</sup> T cells also expressed CD69, suggesting engagement of the T cells with APCs in the lungs; this effect was also CD4<sup>+</sup> T cell independent. The CD8<sup>+</sup> T cells proliferated equally in the presence or absence of CD4<sup>+</sup> T cells, although the BrdU assay performed does not allow one to



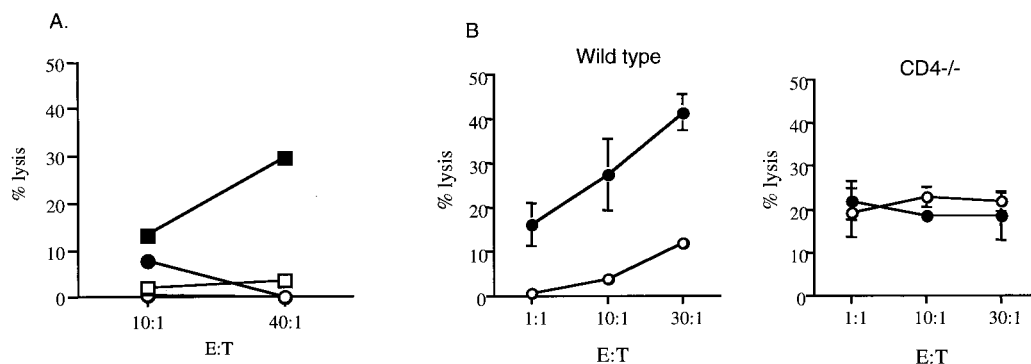
precisely conclude that the replication has occurred in the lungs. The cells may have taken up BrdU at another site (e.g., the lymph node) and trafficked to the lungs in the time between BrdU administration and harvest of the lungs. Taken together, these results indicate that CD4<sup>+</sup> T cells are not required for migration, expansion, and activation of CD8<sup>+</sup> T cells in the lymph nodes and lungs during *M. tuberculosis* infection. Cytokine production by effector CD8<sup>+</sup> T cells in the lungs was also examined. We found that similar percentages of CD8<sup>+</sup> T cells in the lungs of both WT and CD4-deficient mice secreted TNF- $\alpha$  and IFN- $\gamma$  in response to anti-CD3/anti-CD28 Ab stimulation. Our data further indicated that the observed cytokine secretion was at least partially due to the activation of *M. tuberculosis*-specific cells. CD8<sup>+</sup> T cells also appeared to be actively secreting IFN- $\gamma$  in the lungs of both groups of mice, confirming that priming of cytokine-producing effectors was not impaired in the absence of helper cells.

Despite the highly activated phenotype of CD8<sup>+</sup> T cells present in the lungs of CD4-deficient mice, they were impaired in their cytotoxic function. Cultured CD8<sup>+</sup> T cells from lungs of mutant mice failed to lyse infected macrophages. It is possible that CD4<sup>+</sup> T cells are required for in vitro culture of mycobacteria-specific CTLs. CD8<sup>+</sup> T cells from lymph nodes of infected mice cultured under identical conditions had WT levels of lysis of infected macrophages, arguing that CD4 T cells are not necessary during the in vitro culture. However, it is not possible to distinguish in vitro priming of naive lymph node cells from restimulation of previously primed cells in the in vitro culture. The *M. tuberculosis*-specific CD8<sup>+</sup> T cells from the lymph nodes may also not be cytotoxic following in vitro culture, and the lysis observed may be due to those CD8<sup>+</sup> T cells primed in vitro, as we observed using uninfected mice. However, CD8<sup>+</sup> T cells in the lungs of CD4-deficient mice do appear to be deficient in cytotoxic function. Because the antigenic repertoire of *M. tuberculosis*-specific CTLs is not known, it was not possible for us to test the cytotoxic activity of the full spectrum of Ag-specific CD8<sup>+</sup> T cells directly ex vivo. To examine the cytotoxic potential of CD8<sup>+</sup> T lymphocytes without in vitro cell culturing, we used a redirected target lysis assay. CD8<sup>+</sup> T cells freshly harvested from lungs of infected CD4<sup>-/-</sup> mice failed to induce Ab-redirected target lysis. The failure of CD8<sup>+</sup> T cells to exert cytotoxicity was not due to the general unresponsiveness of these cells, as they readily secreted cytokines upon TCR triggering. A similar situation was recently reported to occur during chronic LCMV infection in the absence of CD4<sup>+</sup> T cells (59). In these studies, the presence of activated (CD69<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup>) CD8<sup>+</sup> T cells during infection was

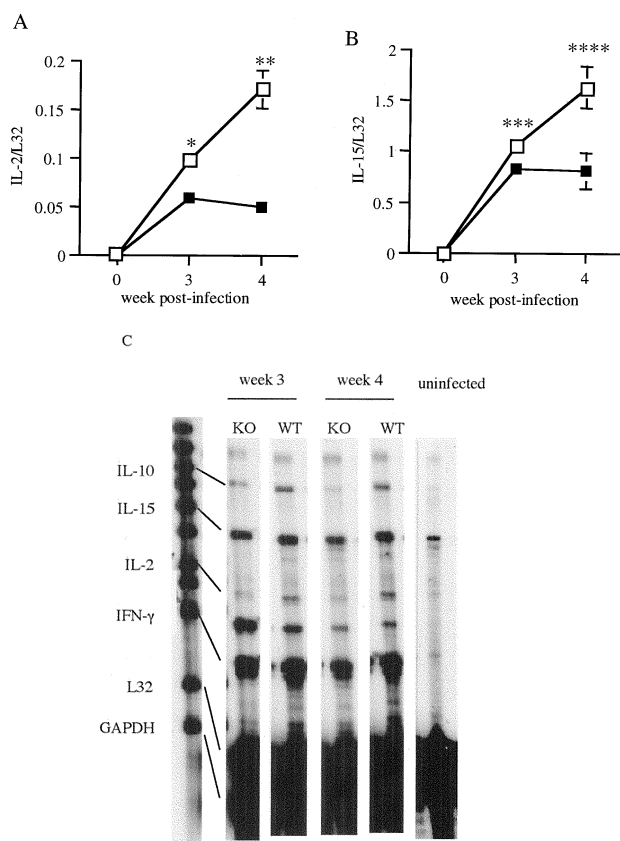
observed under conditions of CD4 T cell deficiency. Although these virus-specific CD8<sup>+</sup> T cells expressed activation markers and proliferated in vivo, they were unable to exert effector functions. Recently, Moser et al. (60) described polyoma virus-specific CD8<sup>+</sup> T cells that lacked cytotoxic activity despite intact perforin expression, suggesting that regulation of lytic activity in CD8<sup>+</sup> T cells extends beyond the expression of perforin or granzyme molecules. Preliminary data from ongoing studies in our laboratory suggest that perforin levels are similar between CD8<sup>+</sup> T cells from WT and CD4<sup>+</sup> T cell-deficient mice (data not shown).

One possible explanation for the silencing of the effector responses of CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cell help is the lack of appropriate costimulation by APCs. Our results indicate that absence of CD4<sup>+</sup> T cells might not affect the quality of APC during priming of the immune response to *M. tuberculosis*. However, the effects of CD4<sup>+</sup> T cells on APC function and stimulation of CD8<sup>+</sup> T cell responses during ongoing infection in the lungs remain to be determined. In addition to "conditioning" the APCs during infection, CD4<sup>+</sup> T cells likely function as a source of cytokines, such as IL-2. CD4<sup>+</sup> T cell deficiency in mice is associated with lower IL-2 responses (8); this cytokine is involved in development of cytotoxic responses. We hypothesized that CD4<sup>+</sup> T cells in the lymph nodes and lungs secrete IL-2 and possibly other cytokines that activate and maintain the cytotoxic function of CD8<sup>+</sup> T cells. IL-2 gene expression was diminished in the lungs of CD4-deficient mice throughout the course of infection. Interestingly, the reduced IL-2 levels did not appear to affect the incorporation of BrdU by CD8<sup>+</sup> T cells, suggesting that even low levels of this cytokine are sufficient to sustain proliferative responses in vivo. CD8<sup>+</sup> T cells can make low levels of IL-2 under certain circumstances and may be responsible for the IL-2 expression in the absence of CD4<sup>+</sup> T cells.

The levels of another CTL-activating cytokine, IL-15, were also reduced in the CD4<sup>-/-</sup> lungs as compared with the WT lungs. Although IL-15 production is attributed to nonlymphoid cells such as monocytes and DCs (61, 62), optimal IL-15 production might require interaction of these cells with CD4<sup>+</sup> T cells, as was shown for IL-18 production by macrophages from tuberculosis patients (63). IL-15 has been shown to enhance and restore CD8<sup>+</sup> T cell responses in *Toxoplasma gondii*-infected or immunized mice (31, 32). In addition, exogenous administration of IL-15 enhanced the ability of mice to control *M. tuberculosis* infection (64), although CD8<sup>+</sup> T cell responses were not assessed. In our system, diminished CTL responses in the absence of CD4<sup>+</sup> T cell help might be



**FIGURE 6.** Cytotoxic activity of CD8<sup>+</sup> T cells from lungs of WT and CD4<sup>-/-</sup> mice. **A**, Lung cells were harvested from WT (squares) and CD4<sup>-/-</sup> (circles) mice 4 wk postinfection (i.v.), cultured with *M. tuberculosis*-infected MHC class II<sup>-/-</sup> DC for 5 days, and used in a 4-h <sup>51</sup>Cr release cytotoxicity assay. Targets: uninfected macrophages (open symbols) and macrophages infected for 36 h (filled symbols). **B**, Lung cells were harvested from 4-wk-infected WT (left panel) and CD4<sup>-/-</sup> (right panel) mice and used directly in a 4-h <sup>51</sup>Cr release assay against FeR<sup>+</sup> P815 cells in the absence (○) or presence (●) of anti-TCR $\alpha\beta$  Ab. WT mice were depleted of CD4<sup>+</sup> T cells 4 days before lung harvest. The experiment was repeated two times. Error bars represent SE.



**FIGURE 7.** Expression of IL-2 and IL-15 genes in the lungs of WT and CD4<sup>-/-</sup> mice during *M. tuberculosis* infection. Mice were infected via aerosol and lungs were harvested 0, 3, and 4 wk postinfection. Total RNA was isolated and expression of IL-2 (A) and IL-15 (B) genes was examined using the ribonuclease protection assay. Intensities of the bands on the autoradiographs were quantitated by densitometry and the results are expressed as ratio of band intensities of genes of interest and housekeeping gene, L32. Each time point represents two to four mice. Error bars represent SE. The experiment was repeated once with similar results. \*,  $p < 0.001$ ; \*\*,  $p = 0.03$ .

due to a deficient cytokine environment. This hypothesis is consistent with our observation that, in contrast to cytotoxic function, cytokine production by CD8<sup>+</sup> T cells is unimpaired in CD4-deficient mice. Recently, HIV-specific clones were reported to be deficient in cytotoxic activity while still retaining the ability to secrete cytokines (65). Thus, the mechanism by which CD4<sup>+</sup> T cells regulate CD8<sup>+</sup> T cell function might be common to both viral and bacterial pathogens.

CD8<sup>+</sup> T cell effector function in the absence of CD4<sup>+</sup> T cell help has been studied mostly in viral systems, and the mechanisms underlying the dependence of CD8<sup>+</sup> T cells on CD4<sup>+</sup> T cell help are not completely clear. To our knowledge, this is the first study that examines in detail the effect of CD4<sup>+</sup> T cell deficiency on the effector functions of CD8<sup>+</sup> T cells during bacterial infection. In summary, we have demonstrated that a CD4<sup>+</sup> T cell deficiency severely impaired the development of cytotoxic CD8<sup>+</sup> T cell responses during *M. tuberculosis* infection while having no discernible effect on the priming, migration, and activation of this cell subset in the lymph nodes and lungs. In contrast to cytotoxic function, cytokine production by CD8<sup>+</sup> T cells was not obviously affected by the absence of CD4<sup>+</sup> T help, suggesting differential regulation of the development of these effector functions. The lack of CD4<sup>+</sup> T cells resulted in diminished levels of IL-2 and IL-15, which may contribute to reduced effector functions of CD8<sup>+</sup> T

cells in the lungs. The absence of CD4<sup>+</sup> T cells in both acute and chronic tuberculosis results in a fatal course of infection, even though there are CD8<sup>+</sup> T cells capable of producing high levels of IFN-γ. This supports the hypothesis that CD4<sup>+</sup> T cells have important, nonredundant roles in control of *M. tuberculosis* in addition to IFN-γ production. The data presented in this work suggest that CD4<sup>+</sup> T cells provide help for the development of cytotoxic CD8<sup>+</sup> T cell populations and the cytotoxicity exerted by effector CD8<sup>+</sup> T cells might be an important component of antimycobacterial immunity. These findings are relevant with respect to the development or maintenance of immune responses under conditions of immunodeficiency, such as HIV infection, when CD4<sup>+</sup> T cells are depleted. The bacterial load and numbers of functional CD4<sup>+</sup> T cells may be important factors in determining whether successful cytotoxic or cytokine-producing CD8<sup>+</sup> T cells are primed and maintained during a bacterial infection such as tuberculosis. The effect of diminished CD4<sup>+</sup> T cells on the development or maintenance of a CD8<sup>+</sup> T cell memory response in tuberculosis is also unclear. Because HIV infection and the subsequent loss of CD4 T cells are major risk factors for development of active tuberculosis, these questions are important for vaccine and therapeutic approaches against tuberculosis.

## Acknowledgments

We thank Heather Joseph and Holly Scott for assistance with the ribonuclease protection assays and Dr. Walter Storkus for providing cytokines for DC culture. We are also grateful to Dr. John Chan and the members of the Flynn laboratory for helpful discussions and to Dr. Robert Hendricks for careful reading of the manuscript.

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