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# Activation of CD8 T Cells by Mycobacterial Vaccination Protects against Pulmonary Tuberculosis in the Absence of CD4 T Cells<sup>1</sup>

Jun Wang, Michael Santosuosso, Patricia Ngai, Anna Zganiacz, and Zhou Xing<sup>2</sup>

We have investigated whether both primary CD8 T cell activation and CD8 T cell-mediated protection from *Mycobacterium tuberculosis* challenge could occur in mycobacterial-vaccinated CD4 T cell-deficient (CD4KO) mice. Different from wild-type C57BL/6 mice, s.c. vaccination with bacillus Calmette-Guérin (BCG) in CD4KO mice failed to provide protection from secondary *M. tuberculosis* challenge at 3 wk postvaccination. However, similar to C57BL/6 mice, CD4KO mice were well protected from *M. tuberculosis* at weeks 6 and 12 postvaccination. This protection was mediated by CD8 T cells. The maintenance of protective effector/memory CD8 T cells in CD4KO mice did not require the continuous presence of live BCG vaccine. As in C57BL/6 mice, similar levels of primary activation of CD8 T cells in CD4KO mice occurred in the draining lymph nodes at 3 wk after BCG vaccination, but different from C57BL/6 mice, the distribution of these cells to the spleen and lungs of CD4KO mice was delayed, which coincided with delayed acquisition of protection in CD4KO mice. Our results suggest that both the primary and secondary activation of CD8 T cells is CD4 T cell independent and that the maintenance of these CD8 T cells is also independent of CD4 T cells and no longer requires the presence of live mycobacteria. However, the lack of CD4 T cells may result in delayed distribution of activated CD8 T cells from draining lymph nodes to distant organs and consequently a delayed acquisition of immune protection. Our findings hold implications in rational design of tuberculosis vaccination strategies for humans with impaired CD4 T cell function. *The Journal of Immunology*, 2004, 173: 4590–4597.

Pulmonary tuberculosis (TB)<sup>3</sup> caused by *Mycobacterium tuberculosis* and AIDS caused by HIV are the two leading infectious causes of death worldwide. More than one-third of HIV-positive individuals are coinfecting with *M. tuberculosis* and ~12% of AIDS deaths are attributed to TB (1–3). The progressive loss of CD4 T cells in HIV-infected patients is the basis of increased TB incidence since CD4 T cells are considered to be the primary cellular component involved in immune protection against TB via their ability to produce IFN- $\gamma$ , activate macrophages, and kill infected cells (4–9). Furthermore, CD4 T cells are believed to be required either for primary activation of CD8 T cells (10–12) or for the maintenance of immune protective CD8 T cells (13–18) under many immunological conditions. Indeed, there has been a considerable body of literature to support the important role of CD4 T cells in host defense against primary TB infection. However, the questions still remain poorly understood regarding whether CD4 T cells are required for primary activation, upon TB vaccination, and secondary activation, upon secondary *M. tuberculosis* challenge, of CD8 T cells, and whether such activated CD8

T cells, in the absence of CD4 T cells, are capable of immune protection from TB. Studies designed to address these questions hold important implications in the development of vaccination strategies aiming to activate CD8 T cells of immune-deficient hosts such as HIV-infected people who suffer from diminished numbers of CD4 T cells.

The development of effective vaccination strategies for CD4 T cell-deficient hosts entails enhanced understanding of CD8 T cell biology in host defense against intracellular bacterial pathogens. Increasing evidence suggests that in addition to their cytolytic activities, CD8 T cells are also able to release IFN- $\gamma$  (4, 5). Thus, the lack of CD8 T cells as the consequence of gene deficiency in MHC class I, TAP1, or  $\beta_2$ -microglobulin or by Ab-mediated CD8 T cell depletion results in weakened host defense against primary mycobacterial infection regardless of the presence of CD4 T cells (19–27). In contrast, adoptive transfer of CD8 T cells generated in the presence of CD4 T cells from DNA vaccine or bacillus Calmette-Guérin (BCG)-immunized hosts confers significant immune protection against secondary *M. tuberculosis* challenge (28–30). These results suggest that CD8 T cells, when properly activated, have the potential to protect from TB. Thus, in our current study we set out to specifically investigate 1) whether CD8 T cells could be activated in the absence of CD4 T cells by mycobacterial vaccination; 2) whether these CD8 T cells protect the hosts from secondary *M. tuberculosis* challenge in the absence of CD4 T cells; and 3) whether the immune protective potential of these CD8 T cells could be maintained over time postvaccination in the absence of both CD4 T cells and live mycobacterial organisms.

We have used a model involving the use of CD4 T cell-deficient mice and s.c. vaccination with a low dose of BCG inocula to address the above questions. Our results suggest that both the primary (upon BCG vaccination) and secondary activation (upon *M. tuberculosis* challenge) of CD8 T cells is CD4 T cell independent. Once generated, the maintenance of immune protective CD8 T

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<sup>3</sup> Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; LN, lymph node; SLN, subiliac LN; CFP, culture filtrate protein; ICCS, intracellular cytokine staining; wt, wild type; MHC II, MHC class II; c.BGG, crude BCG.

cells is also independent of the presence of live mycobacterial organisms. However, compared with their wild-type (wt) counterparts, immunized CD4 T cell-deficient hosts mounted a delayed immune protection that was associated with delayed distribution of Ag-specific CD8 T cells from local draining lymph nodes (LN) into the spleen and lung.

## Materials and Methods

### Mice

CD4-deficient (CD4KO; C57BL/6 background) mice were originally purchased from Taconic Farms (Germantown, NY) and were bred under the pathogen-free conditions at the Central Animal Facility of McMaster University (Hamilton, Ontario, Canada). Male and female CD4KO mice were used at the age of 6–8 wk. The wt control C57BL/6 mice at the same age were purchased from Harlan Breeders (Indianapolis, IN).

### BCG vaccination, *M. tuberculosis* challenge and colony-forming assay

*Mycobacterium bovis* BCG (Connaught strain) and *M. tuberculosis* (H37<sub>RV</sub> strain) (ATCC 27294) were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with Middlebrook OADC enrichment (Invitrogen Life Technologies, Gaithersburg, MD), 0.002% glycerol, and 0.05% Tween 80 for ~10–15 days, then aliquoted and stored in –70°C until needed. Before each use, BCG or *M. tuberculosis* bacilli were washed with PBS containing 0.05% Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps. For immunization, 10,000 CFU of live BCG in a total volume of 100  $\mu$ l of PBS were s.c. injected into CD4KO mice or C57BL/6 mice around each hind leg as described previously (31, 32). With this dose of BCG, both C57BL/6 mice and CD4 KO mice were able to confine the bacteria at injection site without significant systemic dissemination as assessed by colony enumeration assay in the lung and spleen (see below). For challenge, immunized or nonimmunized mice were infected intranasally with 10,000 CFU of *M. tuberculosis* at indicated time points after immunization in the Level III containment facility of McMaster University. The level of bacterial burden in the lung and spleen was determined at 4 wk after *M. tuberculosis* challenge by plating serial dilutions of tissue homogenates in triplicates onto Middlebrook 7H10 agar plates containing OADC enrichment (32). Plates were incubated inside semisealed plastic bags at 37°C for 21 days, and colonies were counted, calculated, and presented as log<sub>10</sub> CFU per organ.

### Lymphocyte isolation and *in vitro* Ag stimulation assay

CD4KO mice and C57BL/6 mice were sacrificed at various time points after s.c. BCG immunization and spleens and subiliac LN (SLN) draining the site of immunization were removed aseptically and pooled from three to five mice per time point. Cells were isolated and cultured as previously described (31–34). Approximately  $0.5 \times 10^6$  cells were seeded into 96-well plates and cultured in RPMI 1640 medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mmol/L L-glutamine) with or without Ag stimulation at 37°C. The Ags used include crude BCG (1  $\mu$ l/well), *M. tuberculosis* culture filtrate protein (CFP) (4  $\mu$ g/ml), or an irrelevant Ag keyhole limpet hemocyanin (10  $\mu$ g/ml) stimulation. The culture supernatants were collected at 72 h and stored at –20°C until IFN- $\gamma$  measurement.

### Coculture of purified CD8 T cells with APC *in vitro*

In some experiments, CD8<sup>+</sup> T cells were further purified from whole splenocytes and SLN-derived cells at indicated time points following s.c. BCG immunization in C57BL/6 mice by negative selection columns (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The purity of the column is around 85–90% as determined by FACS staining, in which the percentage of CD4 T cells in the preparation is close to zero (data not shown). Approximately  $0.5 \times 10^6$  purified CD8 T cells were cocultured with  $0.5 \times 10^6$  gamma-irradiated splenocytes ( $\gamma$ -APC) that crude BCG (c.BCG) were prepulsed with 1  $\mu$ l of CFP and 1  $\mu$ l of crude BCG (c.BCG) at 37°C. Background controls were also set up, including  $\gamma$ -APC alone,  $\gamma$ -APC with CFP, and c.BCG, purified CD8 T cells alone, and purified CD8 T cells with  $\gamma$ -APC but without CFP and c.BCG stimulation. The culture supernatants were collected at 72 h and stored at –20°C until IFN- $\gamma$  measurement. All of the controls had only minimal background levels of IFN- $\gamma$  and were left out from the figure for the purpose of simplicity.

### FACS analysis, IFN- $\gamma$ intracellular cytokine staining (ICCS) and ELISPOT assay

CD4KO mice and C57BL/6 mice were sacrificed at various time points following s.c. BCG immunization. Single-cell suspensions were prepared from individual spleen, SLN, and lung following collagenase digestion as described previously (33, 34). Approximately  $2 \times 10^6$  cells were seeded into a round-bottom 96-well plate and stimulated with or without 1  $\mu$ l of CFP and 1  $\mu$ l of c.BCG at 37°C for a total of 16 h in the presence of 10  $\mu$ g/ml brefeldin A for the last 4 h. In some experiments, 20 ng/ml PMA and 2  $\mu$ M ionomycin (Sigma-Aldrich, St. Louis, MO) were also added during the last 4 h. The cells were then washed, blocked for 15 min with anti-CD16/CD32 in PBS/0.5% BSA, and then stained with FITC-anti-murine CD4 (clone H129.19) and CyChrome-anti-murine CD8 $\alpha$  (clone 53-6.7; BD Pharmingen, Mississauga, Ontario, Canada) for 30 min on ice. The cells were then washed, fixed, and permeabilized with the reagents provided in an ICCS kit (BD Pharmingen) according to the manufacturer's instructions. The cells were then stained with PE-anti-murine IFN- $\gamma$  or isotype control PE-rat IgG1 for 30 min on ice. Normally, a total of 300,000–500,000 events per sample was collected by FACScan (BD Biosciences, Sunnyvale, CA) and analyzed using WinMDI2.8 software (The Scripps Institute, La Jolla, CA). The ELISPOT assay was set up as previously described (34) and used to evaluate the frequency of Ag-specific T cells.

### *In vivo* depletion of CD8 T cells in CD4KO mice

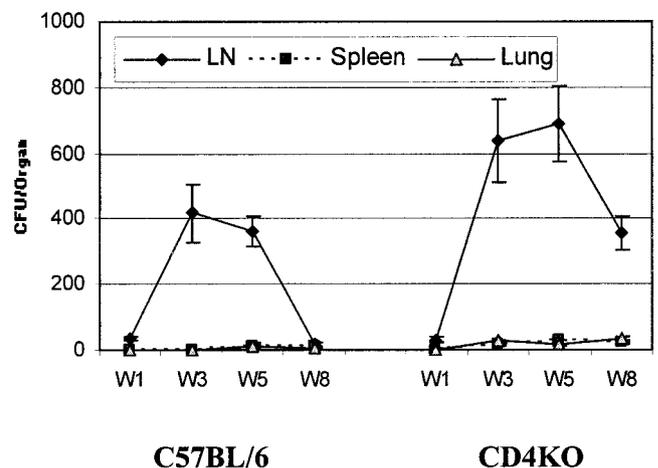
BCG-immunized CD4 KO mice were injected i.p. with anti-mouse CD8 mAb (clone 2.43) (34) at a dosage of ~150  $\mu$ g at 3 days before (day –3), 100  $\mu$ g at 1 day before (day –1) and at the day (day 0) of *M. tuberculosis* challenge, and 100  $\mu$ g once weekly after *M. tuberculosis* challenge until the end of the experiment. As an isotype control, some BCG-immunized CD4KO mice were injected only with anti-CD4 mAb (clone GK1.5) (34).

### Antimycobacterial antibiotic treatment

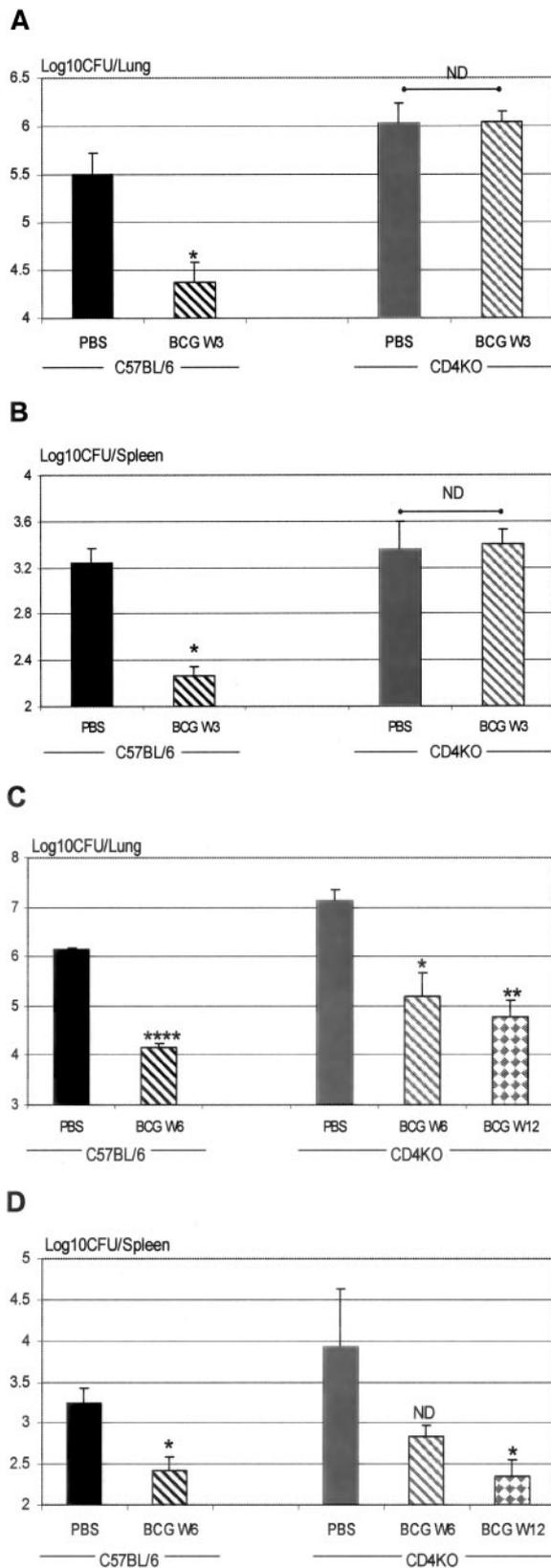
BCG- or PBS-immunized CD4KO mice were treated at 4 wk postimmunization for a period of 4 wk with 25 mg/kg isoniazid daily by gavage. These mice were then challenged with *M. tuberculosis* 2 wk after cessation of antibiotic treatment.

### Cytokine measurements

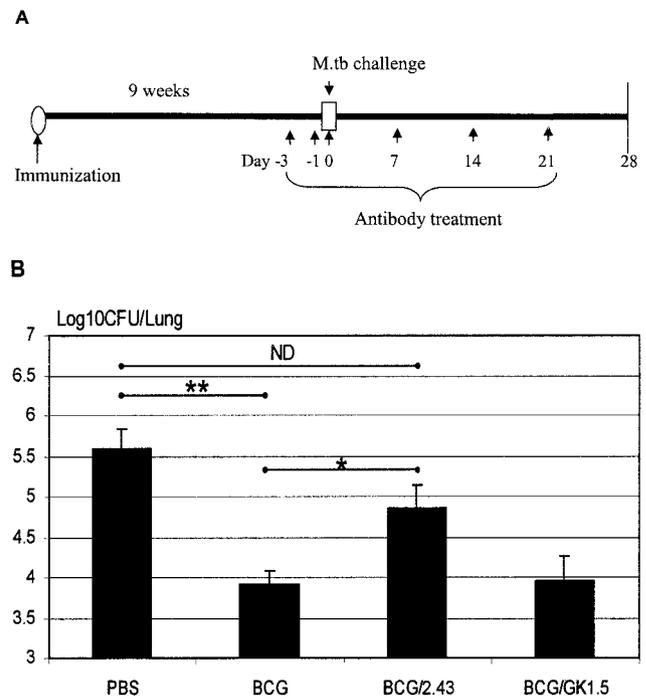
The level of IFN- $\gamma$  in the culture supernatants was measured by using a mouse-specific ELISA kit (R&D Systems). The sensitivity of detection for IFN- $\gamma$  was 2 pg/ml.



**FIGURE 1.** Growth kinetics of BCG in the LN, spleen, and lung following s.c. immunization. C57BL/6 and CD4KO mice were immunized s.c. with 10,000 CFU of BCG and sacrificed at weeks 1, 3, 5, and 8. The number of bacilli in the local draining LN, spleen, and lung was determined by colony enumeration assay. The results are expressed as mean  $\pm$  SEM from four mice per group per time point.



**FIGURE 2.** Immune protection against pulmonary TB in BCG-immunized C57BL/6 and CD4KO mice. CD4KO mice and C57BL/6 mice were immunized s.c. with BCG for 3 wk (A and B), 6 wk (C and D), or 12 wk (C and D) or treated with PBS as a control for 3 wk (A and B) or 6 wk (C and D) and then challenged via the airway with *M. tuberculosis*. The number of bacilli in the lung (A and C) and spleen (B and D) was determined 4 wk postchallenge by colony enumeration assay. The results are expressed as mean  $\pm$  SEM from six to eight mice per group per time point. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.000001$  as compared with PBS-treated control group.



**FIGURE 3.** In vivo depletion of CD8 T cells by using mAb (clone 2.43) in BCG-vaccinated CD4KO mice. A, BCG-immunized CD4KO mice were repeatedly treated with or without mAb 2.43 or control Ab GK1.5 starting from 9 wk after BCG vaccination as detailed in the diagram. These mice were challenged via the airway with *M. tuberculosis* (M.tb). B, Mice were then sacrificed at 4 wk after *M. tuberculosis* challenge and the number of bacteria was enumerated in the lung. The results are expressed as mean  $\pm$  SEM from five to six mice per group. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$  as compared with PBS control.

## Results

### Immune protection against pulmonary tuberculosis in BCG-immunized CD4KO mice

We have previously demonstrated that CD4 T cell-deficient mice were capable of mounting an effective cellular immune response in the lung following pulmonary mycobacterial infection with *M. bovis* BCG, suggesting CD4 T cell-independent CD8 T cell activation in response to mycobacterial infection (24, 27). To address whether s.c. BCG vaccination in CD4KO mice could stimulate CD8 T cell responses and confer protection against *M. tuberculosis* infection, CD4KO mice and wt control C57BL/6 mice were immunized s.c. with 10,000 CFU of BCG. This small dose of inoculum was selected for vaccination since it did not cause systemic dissemination of mycobacteria in CD4 T cell-deficient mice following s.c. inoculation (Fig. 1). Indeed, like in B6 wt control mice, very few bacilli were present in the spleen and lung of CD4 KO mice and the kinetic changes of detectable bacilli in the local draining LN of these mice were similar to those in B6 wt mice although they were at a higher magnitude (Fig. 1). Immunized and nonimmunized mice were then challenged with a virulent strain of *M. tuberculosis* through the airway at weeks 3, 6, and 12 after immunization. Compared with nonimmunized B6 wt mice, BCG-immunized B6 mice were markedly protected from *M. tuberculosis* challenge at both weeks 3 and 6 postimmunization ( $p < 0.05$  for week 3 and  $p < 0.000001$  for week 6; Fig. 2, A–D). In comparison, there was no immune protection detected in BCG-immunized CD4KO mice at week 3 postimmunization (Fig. 2, A and B). However, significant immune protection was observed at weeks 6

and 12 postimmunization with 2–2.4 log reduction of *M. tuberculosis* infection in the lung compared with the nonimmunized CD4KO control ( $p < 0.05$  for week 6 and  $p < 0.001$  for week 12; Fig. 2, C and D). And the level of protection in CD4KO mice was comparable to that achieved in B6 wt control mice. These results suggest that compared with wt mice, BCG vaccination triggers a delayed but sustained immune protection in the hosts that lack CD4 T cells.

#### CD8 T cell-mediated immune protection against pulmonary TB in BCG-immunized CD4KO mice

To examine whether the protective immunity in BCG-immunized CD4KO mice was mediated directly by CD8 T cells, BCG-immunized CD4KO mice were repeatedly treated with or without anti-CD8 mAb or control Ab starting at 9 wk postimmunization (Fig. 3A) and the level of immune protection against *M. tuberculosis* challenge was compared. In keeping with the previous experiments (Fig. 2), BCG-immunized CD4KO mice at week 9 without CD8 T cell depletion were protected from *M. tuberculosis* challenge with ~1.7 log reduction in the level of infection in the lung (Fig. 3B). Also, BCG-immunized CD4KO mice receiving the isotype control anti-CD4 Ab treatment were also well protected, similar to the BCG-immunized CD4KO mice without Ab treatment (Fig. 3B). In contrast, the mice depleted of CD8 T cells had a significantly reduced level of protection in the lung (at least 60% reduced; Fig. 3B). These results suggest that immune protection achieved by BCG immunization in CD4KO mice is attributed largely to CD8 T cells and the secondary activation of these CD8 T cells, upon *M. tuberculosis* challenge, does not require CD4 T cells.

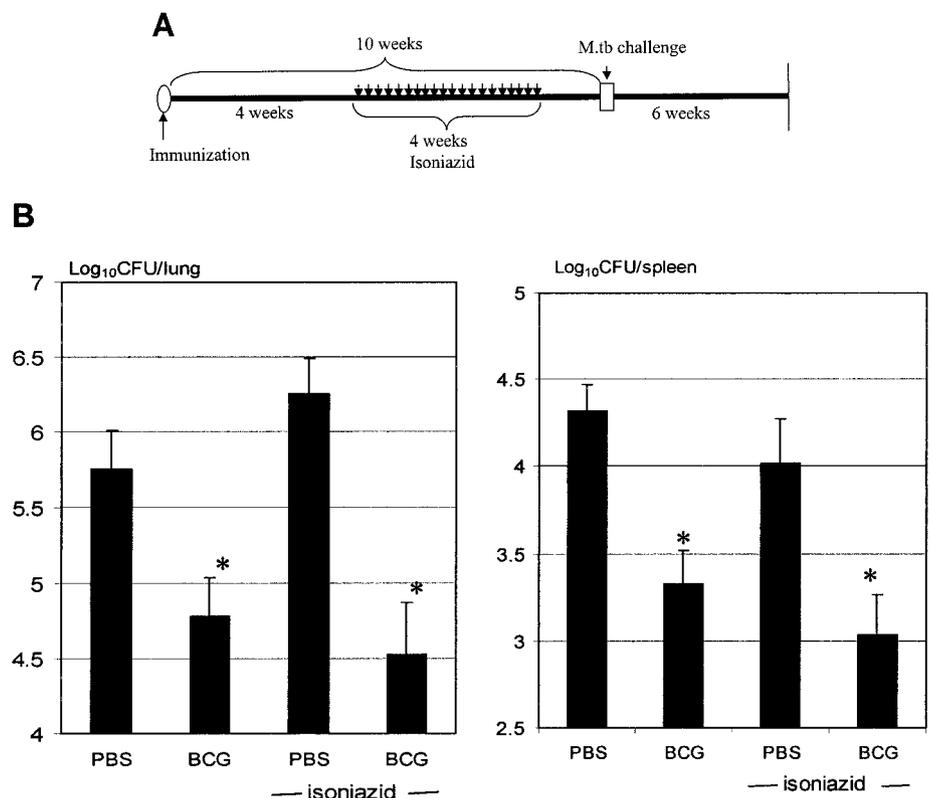
#### Maintenance of memory immune protection does not require the continuous presence of live mycobacterial organisms in CD4KO mice

We have found that although BCG bacilli were primarily localized in the draining LN after s.c. BCG inoculation in both wt B6 and

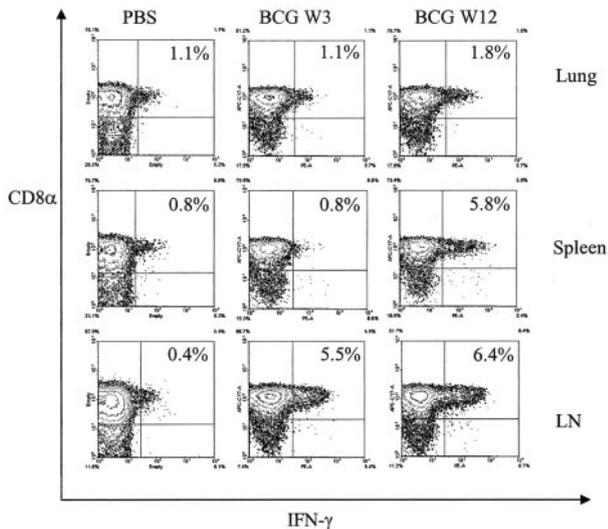
CD4KO mice, there appeared to be delayed clearance or persistence of mycobacteria in CD4KO hosts (Fig. 1). Thus, we set out to address whether immune protection of BCG-immunized CD4KO mice could be mediated by authentic effector/memory CD8 T cells or was dependent on the continuous generation of effector CD8 T cells and the persistence of BCG bacilli in the host. To this end, we treated BCG-immunized CD4KO mice daily with a potent antimycobacterium antibiotic, isoniazid, starting at 4 wk after BCG vaccination for a period of 4 wk. These mice were then challenged with *M. tuberculosis* 2 wk after cessation of isoniazid (Fig. 4A). As an internal control, some nonimmunized PBS-treated mice were also treated daily with isoniazid before challenge. All mice were sacrificed at 6 wk postchallenge. The level of TB infection in PBS control group was high (Fig. 4B). Likewise, the level of infection in the antibiotic-treated PBS control group was similar to that in the non-antibiotic-treated PBS control group (Fig. 4B), suggesting that isoniazid was cleared at the time of *M. tuberculosis* challenge and had little effect by itself on the level of TB infection. In contrast, at this point postchallenge, BCG-immunized CD4KO mice with no antibiotic treatment were still well protected (Fig. 4B), and similar levels of protection were also found in BCG-immunized CD4KO mice that received antibiotic treatment. These results suggest that persistence of live organisms is not required to maintain protective immunity in BCG-immunized CD4-deficient hosts and likely such protection is mediated at least in part by memory CD8 T cells.

#### CD4 T cell-independent CD8 T cell activation and the impact of CD4 T cells on the distribution of CD8 T cells in lymphoid and lung tissues

To understand the potential mechanisms underlying the delayed acquisition of protection triggered by BCG immunization in CD4KO mice, we examined the cellular immune activation in the local draining LN where primary immune activation took place and in the distal organs, the lung and spleen to which activated T



**FIGURE 4.** BCG-induced immune protection in CD4KO mice was not dependent on the continuous presence of live mycobacterial organisms. *A*, One-half of PBS-treated or BCG-vaccinated CD4KO mice, starting from the fifth week, were treated daily by gavage with isoniazid for a period of 4 wk. Mice were then challenged with *M. tuberculosis* (M.tb) 2 wk after cessation of the antibiotic regimen to allow the clearance of antibiotic. *B*, Mice were then sacrificed at week 6 postchallenge and the number of colonies in the lung and spleen was determined by colony enumeration assay. Data are expressed as mean  $\pm$  SEM from seven to eight mice per group. \*,  $p < 0.05$  as compared with PBS control.

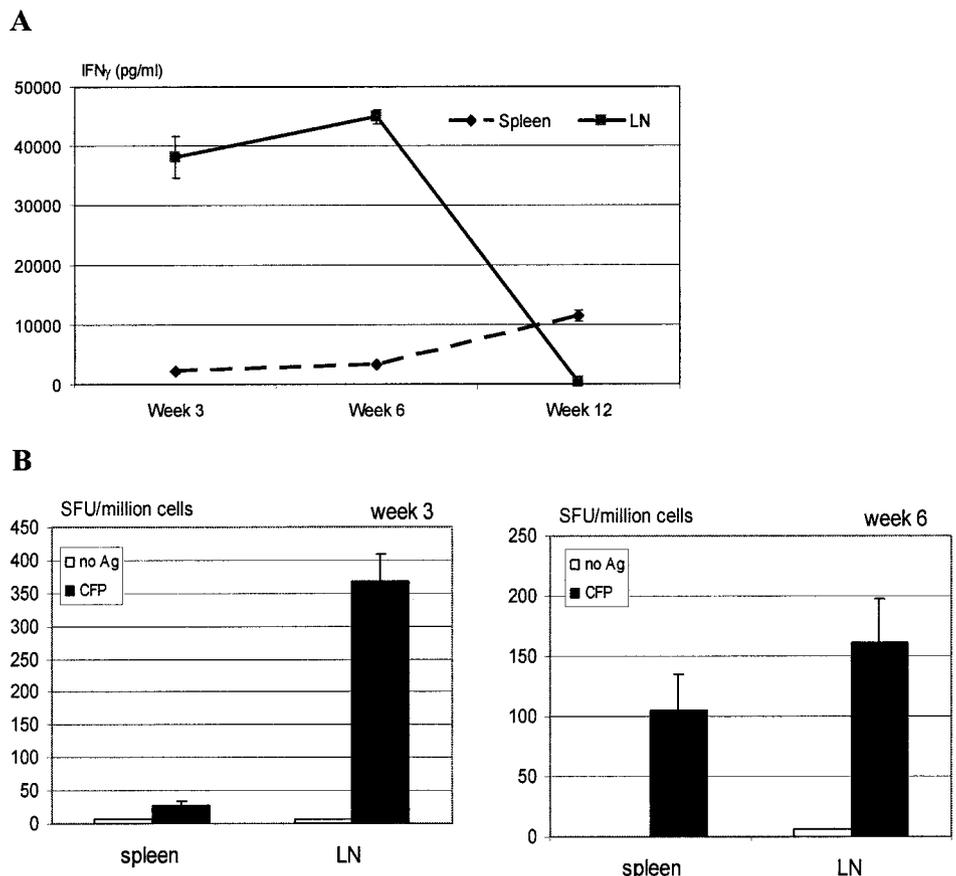


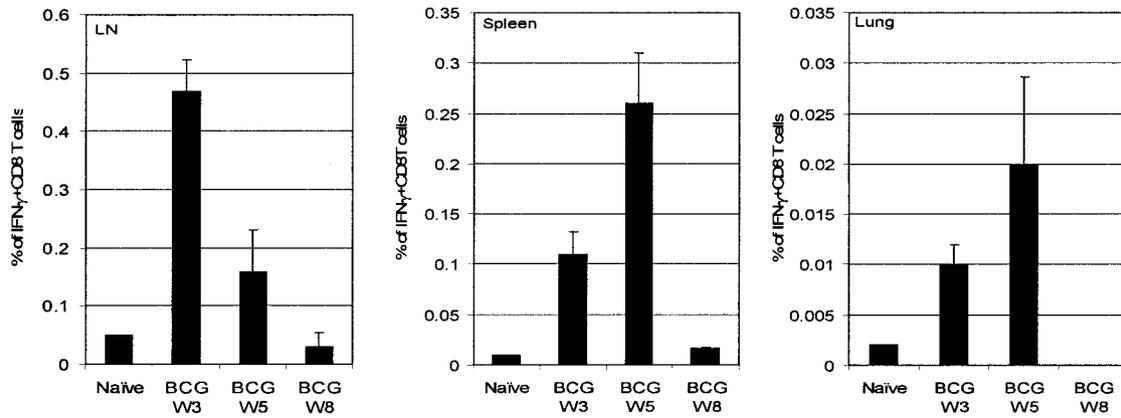
**FIGURE 5.** Delayed CD8 T cell IFN- $\gamma$  responses in the lung and spleen following BCG immunization in CD4KO mice determined by ICCS assay. CD4KO mice were immunized s.c. with BCG or treated with PBS and sacrificed at weeks 3 and 12 postimmunization. The single mononuclear cells were prepared from lungs, spleens, and LN pooled from three mice per time point and stimulated with mycobacterial Ags for 12 h and then further stimulated with PMA and ionomycin in the presence of brefeldin A for an additional 4 h. Cells were then processed for IFN- $\gamma$  ICCS and FACS analysis. The number in the upper right quadrant in each panel represents the percentage of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells of the total lymphocytes gated.

cells redistribute after primary immune activation. We examined this first by using ICCS techniques and chose to focus on two time points postimmunization, week 3 when there was a lack of pro-

tection in CD4KO mice and week 12 when these mice were well protected. Although an increased number of Ag-specific IFN- $\gamma$ <sup>+</sup> CD8 T cells were easily detected in the LN (5.5%) of BCG-immunized CD4KO mice at 3 wk, there were only background IFN- $\gamma$ <sup>+</sup> CD8 T cells detected in the spleen and lung (1.1% for lung and 0.8% for spleen; Fig. 5). In contrast, by 12 wk after BCG immunization, significant numbers of Ag-specific IFN- $\gamma$ <sup>+</sup> CD8 T cells were found not only in LN (6.4%) but also in the lung (1.8%) and spleen (5.8%) of CD4KO mice (Fig. 5). Since not only mycobacterial Ags but polyclonal stimuli were used to stimulate T cells for ICCS assay, we further compared, by using in vitro Ag recall and ELISA (only using mycobacterial Ags for stimulation), immune responses elicited by BCG vaccination in the LN and spleen of CD4KO mice at weeks 3, 6, and 12 postimmunization. The levels of Ag-specific IFN- $\gamma$  responses in the LN-derived cells were high (38,000–55,000 pg/ml) at both weeks 3 and 6 and then declined markedly by week 12 (Fig. 6A). In comparison, the overall kinetic trend of Ag-specific IFN- $\gamma$  responses in the spleen was found to be opposite to those of the LN cells with ~2,000, 3,000, and 12,000 pg/ml IFN- $\gamma$  production measured at weeks 3, 6, and 12 post-vaccination, respectively (Fig. 6A), thus, in basic agreement with the ICCS data (Fig. 5). IFN- $\gamma$  measured in these cultures was found to be released primarily from CD8 T cells since depletion of CD8 T cells by using an anti-CD8 mAb resulted in >90% reduction of IFN- $\gamma$  production (data not shown). We also found that the relative lack of IFN- $\gamma$  responses in CD8 T cells by ELISA was accompanied by lower frequencies of IFN- $\gamma$ -releasing cells at early time points as assessed by the ELISPOT assay (Fig. 6B). Thus, the above data together indicate that CD8 T cell activation in the draining LN upon BCG vaccination could occur independent of CD4 T cells; the accumulation of Ag-specific CD8 T cells in the

**FIGURE 6.** Delayed CD8 T cell IFN- $\gamma$  responses in the spleen following BCG immunization in CD4KO mice. CD4KO mice were immunized s.c. with BCG for 3, 6, or 12 wk and single-cell suspensions from individual mice were prepared from spleen or local draining LN. Cells were cultured with or without mycobacterial Ag stimulation for 72 h, and the level of IFN- $\gamma$  content was measured by ELISA (A) or cells were cultured for 24 h and the number of IFN- $\gamma$ -releasing cells (spot-forming units, SFU) was determined by ELISPOT assay (B). The results are expressed as mean  $\pm$  SEM from triplicate wells.

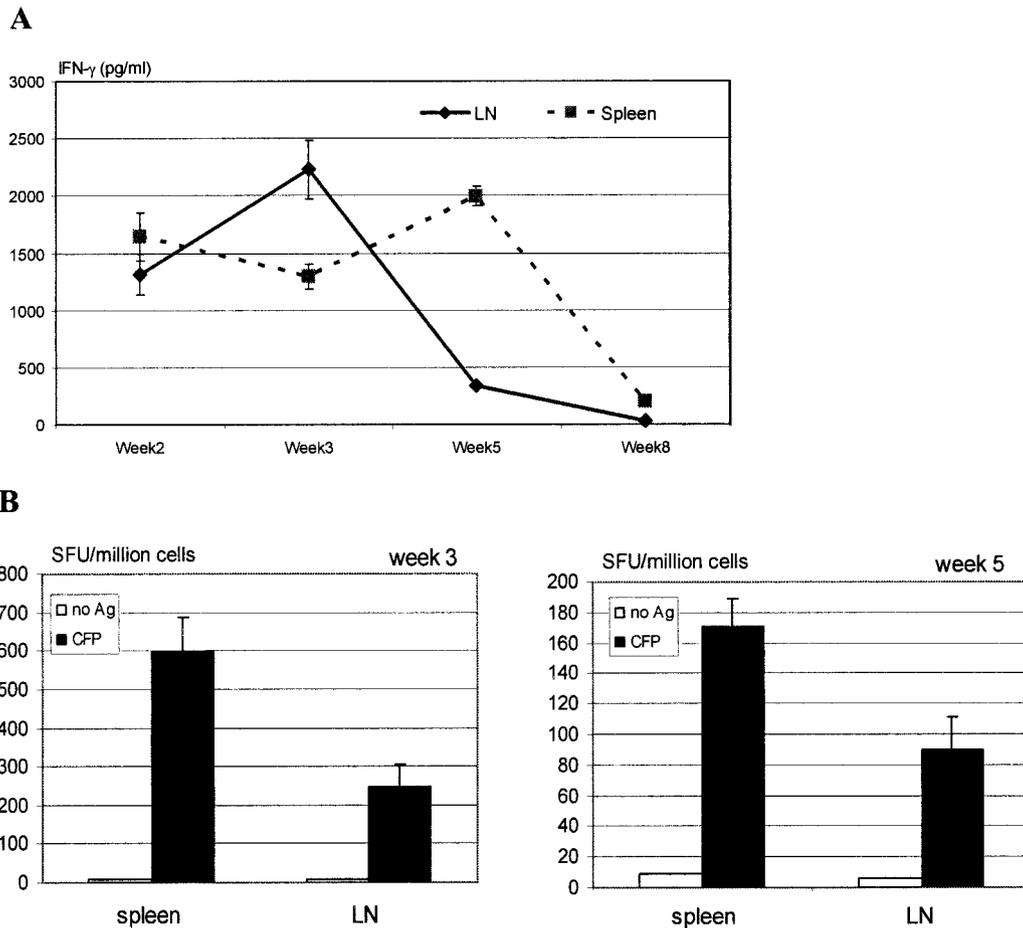




**FIGURE 7.** Kinetics of CD8 T cell activation in the local draining LN, spleen, and lung in wt C57BL/6 mice was determined by ICCS assay. C57BL/6 mice were immunized s.c. with BCG and single mononuclear cell suspensions from individual mice were prepared from LN, spleen, and lung at various time points as indicated (three to four mice per time point) and stimulated with mycobacterial Ags for a total of 16 h (without PMA and ionomycin) for ICCS and FACS analysis. Data are expressed as mean ± SEM of the percentages of CD8<sup>+</sup>IFN-γ<sup>+</sup> cells of the total cells of each organ at a given time point.

spleen and lung of CD4KO mice, however, significantly lags behind that in the LN and such appearance of CD8 T cells in the spleen and lung is closely associated with acquisition of immune protection at later times after BCG immunization in CD4KO mice.

To understand whether the delayed appearance of Ag-specific CD8 T cells in the spleen and lung following BCG immunization was related to the lack of CD4 T cells, we compared the kinetics of CD8 T cell responses in the local draining LN, spleen, and lungs



**FIGURE 8.** Kinetics of CD8 T cell activation in the local draining LN and spleen in wt C57BL/6 mice. C57BL/6 mice were immunized s.c. for various periods of time as indicated. LN and spleens were pooled from four mice per time and CD8 T cells were purified LN cells and splenocytes. Purified CD8 T cells ( $5 \times 10^5$ /well) were cocultured with gamma-irradiated splenocytes and stimulated with mycobacterial Ags for 3 days. The level of IFN-γ content was measured by ELISA (A). In some experiments, total LN cells and splenocytes were stimulated without or with mycobacterial Ags in the presence of anti-CD4 mAbs for 24 h and the number of IFN-γ-releasing CD8 T cells was determined by ELISPOT assay (B). The results are expressed as mean ± SEM from triplicate wells. SFU, Spot-forming units.

that we have found in CD4KO mice with those in wt C57BL/6 mice. Since wt mice had both CD4 and CD8 T cells, we used two different approaches to study CD8 T cell activation. Using ICCS techniques, we determined the percentage of IFN- $\gamma$ <sup>+</sup> CD8 T cells at weeks 3, 5, and 8 after BCG immunization. Different from what we found in CD4KO mice (Figs. 5 and 6), IFN- $\gamma$ <sup>+</sup> CD8 T cells were readily detected in the LN, spleen, and lungs as early as 3 wk postimmunization (Fig. 7). By using a coculture system whereby purified CD8 T cells from LN and spleens were stimulated with APCs, we observed that consistent with ICCS data (Fig. 7), a similar level of IFN- $\gamma$  production released from activated CD8 T cells was detected both in the LN and spleen (Fig. 8A). And again high levels of IFN- $\gamma$  production by CD8 T cells both in the LN and spleen were echoed by markedly increased frequencies of IFN- $\gamma$ -releasing CD8 T cells at early times as assessed by the ELISPOT assay (Fig. 8B). Thus, these findings, along with the observations made in CD4KO mice, suggest that although primary CD8 T cell activation is independent of CD4 T cells, the lack of CD4 T cells may influence the distribution of Ag-specific CD8 T cells from the draining LN to the systemic compartments including the spleen and lung.

## Discussion

The current dreadful TB epidemic in HIV-infected populations has urged the development of efficacious TB vaccines that are suitable for CD4 T cell-deficient hosts. In our current study, we have addressed the question whether functionally immune protective CD8 T cells could be generated in the absence of CD4 T cells by BCG vaccination. Our results suggest that both the primary and secondary activation of CD8 T cells does not require CD4 T cells and, once generated, the maintenance of immune protective CD8 T cells is also independent of the presence of live mycobacterial organisms. However, compared with their wt counterparts, BCG-immunized CD4 T cell-deficient hosts mounted a delayed immune protection which was associated with delayed distribution of Ag-specific CD8 T cells from local draining LN onto the spleen and lung. It is noteworthy that in our model there appeared to be an overactivation of CD8 T cells in CD4KO mice in terms of IFN- $\gamma$  production capacity and frequency, perhaps due to compensatory mechanisms. This may have contributed to the immune protective potential of CD8 T cells in this model and this aspect may be different from that in HIV hosts who generally have depressed CD8 T cell functions (3). Furthermore, given that the current BCG vaccine has failed to control adult TB in humans and it has also been considered unsafe for immune-compromised hosts (6), it would be premature to assume that our current observation supports the ultimate use of BCG vaccine in CD4 T cell-deficient humans. Notwithstanding, we believe that our study provides important evidence that appropriate antigenic exposure could lead to the generation of immune protective CD8 T cells in the absence of CD4 T cells and that such knowledge will help the future design of TB vaccines for such immune-compromised hosts as HIV hosts.

The contribution of CD8 T cells in host defense has been a long-standing debatable subject of TB research. Previously, most experimental designs in dissecting the role of CD8 T cells were conducted in primary mycobacterial infection models in either gene knockout mice or mice receiving CD4- or CD8 T cell-depleting mAbs (19–27). Depending on the experimental settings in terms of the dosage, route, and the strain of bacteria used for the infection, it has been claimed that the contribution of CD8 T cells is very important (23, 25), less important (19, 21, 24, 26), or not important at all (20, 22) in host defense against primary mycobacterial infection. However, the primary infection model is inappropriate to address the protective potential of CD8 T cells from a

vaccination point of view. In this study, we have provided experimental evidence to demonstrate that, when the host is pre-equipped by means of vaccination with functional CD8 effector/memory T cells in the lung/spleen (the presence of these cells in the LN fails to do so), CD4 T cell-deficient hosts are capable of a significant level of immune protection against *M. tuberculosis* challenge at least for the period of observation.

Whether CD4 T cells are required for the primary activation of CD8 T cells and the maintenance of memory CD8 T cells in anti-TB immunity has remained incompletely understood. Recent studies using the pathogens that only cause acute infection suggest that although CD4 T cells may not be required for the primary CD8 T cell activation, they are required for the maintenance of memory CD8 T cells and, upon challenge, the secondary activation of these cells (16–18). In our study, we have found that CD8 T cells in CD4KO mice can be activated to a similar extent as those in wt controls in the draining LN at 3 wk after BCG vaccination, suggesting that CD4 T cells are not required for the primary activation of CD8 T cells. Furthermore, BCG-vaccinated CD4KO mice, when challenged at weeks 6 and 12 postvaccination, were protected similarly as wt controls, suggesting that CD4 T cells are not required for maintaining memory CD8 T cells or for secondary activation of these cells. Thus, depending on the nature of microbes, CD4 T cells may be (16–18) or not be, as in our current study, required for the maintenance of memory CD8 T cells and secondary activation of these cells. It is of importance to realize that BCG vaccine used in our study differ from those (viruses or *Listeria*) used in published studies (16–18) in that it causes a chronic intracellular infection, which may provide sufficient signals for CD8 T cells to bypass the help of CD4 T cells. Indeed, we can still recover a significant number of bacilli from a single LN in CD4KO mice at 8 wk after BCG vaccination. However, as we have shown here, the continuous presence of live BCG vaccine was not required for maintaining such Ag-specific CD8 T cells. The important difference that we have identified in our study between CD4KO and wt mice, however, is that activated CD8 T cells in the draining LN of CD4KO mice seem to have the impaired ability, at early times of primary immune activation, to get distributed over to the important distant organs including both the spleen and lung. We strongly believe that the delayed immune protection of CD4KO mice from *M. tuberculosis* challenge is attributed to the delayed appearance of Ag-specific CD8 T cells in the lung, which is the route of TB infection. By monitoring CD8 cytolytic activity, Serbina and colleagues (13) have also observed an impaired CD8 CTL activity in the lung but not in the local draining LN of CD4KO mice at 4 wk following primary pulmonary *M. tuberculosis* infection. Similarly, the divergent requirement of CD4 T cells at different phases of development of CD8 T cell responses has also been demonstrated in mice that were vaccinated with a tumor vaccine genetically engineered to secrete GM-CSF, live *Listeria monocytogenes*, and malaria Ags (35–37). It is generally believed that T cells acquire a new set of adhesion molecules and chemokine receptors to obtain changes in their homing patterns following initial priming/activation in the local draining LN (38–40). In our current study, although the underlying mechanisms responsible for delayed distribution of CD8 T cells of CD4KO mice from LN to other sites, including the spleen, remain to be elucidated, we did find that compared with B6 wt control, less CD62L<sup>+</sup> CD8 T cells in the local draining LN of CD4KO mice expressed T cell activation surface marker CD44 at the early time after BCG vaccination (data not shown). It is likely that CD4 T cells are required for activated CD8 T cells to switch their LN-homing potential to distribute to the peripheral tissues. Finally, it has recently been reported that a substantial fraction of

CD8 T cells in lymphocytic choriomeningitis virus- or *Listeria*-infected CD4KO but not in acutely CD4 T cell-depleted mice is MHC class II (MHC II) restricted (41). Although it remains unclear whether this is also the case in such chronic intracellular infection models as our current BCG infection model, we cannot rule out that part of the protective CD8 T cells that we have observed in BCG-vaccinated CD4KO mice were activated via a MHC II pathway. Although completely unknown at this point whether there are such MHC II-restricted CD8 T cells in humans chronically depleted of CD4 T cells, the vaccine formulations designed for these human populations ought to contain both MHC I and MHC class II epitopes.

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