Generation of CD8 T Cell Memory Is Regulated by IL-12

Erika L. Pearce and Hao Shen

J Immunol 2007; 179:2074-2081; doi: 10.4049/jimmunol.179.4.2074
http://www.jimmunol.org/content/179/4/2074

References  This article cites 44 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/179/4/2074.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription  Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Generation of CD8 T Cell Memory Is Regulated by IL-12

Erika L. Pearce2 and Hao Shen3

Various signals during infection influence CD8 T cell memory generation, but these factors have yet to be fully defined. IL-12 is a proinflammatory cytokine that has been shown to enhance IFN-γ-producing T cell responses and has been widely tested as a vaccine adjuvant. In this study, we show that IL-12-deficient mice generate a weaker primary CD8 T cell response and are more susceptible to *Listeria monocytogenes* infection, but have substantially more memory CD8 T cells and greater protective immunity against reinfection. Kinetic analyses show that in the absence of IL-12 there is a reduced contraction of Ag-specific CD8 T cells and a gradual increase in memory CD8 T cells as a result of increased homeostatic renewal. By signaling directly through its receptor on CD8 T cells, IL-12 influences their differentiation to favor the generation of fully activated effectors, but hinders the formation of CD8 T cell memory precursors and differentiation of long-term CD8 T cell memory. These results have implications for understanding memory T cell development and enhancing vaccine efficacy, and offer new insight into the role of IL-12 in coordinating the innate and adaptive immune response. *The Journal of Immunology*, 2007, 179: 2074–2081.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research was supported by the National Institutes of Health Grant AI45025 (to H.S.).

2 Current address: Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104

3 Address correspondence and reprint requests to Dr. Hao Shen, Department of Microbiology, University of Pennsylvania School of Medicine, 3610 Hamilton Walk, Philadelphia, PA 19104. E-mail address: hshen@mail.med.upenn.edu

Materials and Methods

**Mice**

C57BL/6 (WT) and B6.129S1-Cr (Ly5.1−/−) mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 (WT), C57BL/6-Il12atm1Jm (p35−/−), C57BL/6-Ilt2tm1Jm (p40−/−), B6.129S1-H12rb2tm1Jm (IL-12Rα2−/−), and OT-I mice were purchased from The Jackson Laboratory. All animals were cared for according to the Animal Care Guidelines of the University of Pennsylvania.
Immunizations

Age-matched mice were immunized i.v. with a sublethal dose of $3 \times 10^4$ CFU of rLmOVA or $1 \times 10^5$ CFU of rLmOVA deleted for actA (ΔactA rLmOVA). For secondary immunizations, mice were challenged i.v. with $1 \times 10^6$ CFU of rLmOVA or with $1 \times 10^7$ CFU ΔactA rLmOVA. Acute infections were resolved and bacteria were cleared within 7–14 days in WT and $p35^{-/-}$ mice. CFUs per spleen and liver were determined 3 days after infection as described previously (25).

BrdU incorporation

Mice were injected with 1 mg of BrdU i.p. at day 45 postinfection and were then fed BrdU in their drinking water for 14 days at a concentration of 0.8 mg/ml until sacrificed.

Ampicillin treatment

Mice were fed ampicillin at a concentration of 2 mg/ml in their drinking water starting on day 14 postinfection until sacrificed.

Flow cytometry and intracellular cytokine staining

All fluorochrome-conjugated mAbs were purchased from BD Pharmingen with the exception of anti-IL-7Rα (anti-CD127), which was purchased from eBioscience. Surface staining and intracellular cytokine staining were performed as previously described (26). For ex vivo intracellular cytokine staining, splenocytes were cultured at 37°C for 5 h in complete medium supplemented with 50 U/ml recombinant human IL-2/1.0 μl/ml GolgiStop in either the presence or absence of OVA257–264 peptide at 1.0 μg/ml. OVA-specific CD8+ T cells were also quantified by direct staining with H2-Kb/OVA257–264 (Kb/OVA) MHC/peptide tetramers. All cells from in vitro cultures were restimulated with PMA (50 ng/ml) and ionomycin (50 ng/ml) 2 h before analysis. GolgiStop was only added for intracellular cytokine staining at 1.0 μl/ml 2 h before analysis. For intracellular BrdU staining, the protocol outlined in the BD Pharmingen BrdU Flow Kit was followed (catalog no. 559619). Intracellular levels of ROS were analyzed by FACS using dichlorofluorescein diacetate (DCFDA; Molecular Probes) as a fluorescent probe (27). Cells were loaded with 5 μM DCFDA for 30 min at 37°C and incubated with DCFDA continuously throughout the experiment. Cells were stained for surface molecules before FACS analysis (27). All FACS plots are from a representative mouse from each group and data in all plots are the average of each group. All error bars indicate SD unless percentages have been calculated, where bars represent SE. Values of $p$ were calculated using Student’s $t$ test. All experiments were performed at least twice with three to five mice per group.

In vitro cell stimulation

Splenocytes from C57BL/6 (WT), B6.Ly5.1/Cr (Ly5.1+ WT), C57BL/6-H122b2m12m1 (p35+/−), C57BL/6-H122b2m12m1 (p40−/−), and B6.129S1-H12rb2m12m1/J (IL-12Rβ2−/−) mice were stimulated in vitro using soluble anti-CD3 and anti-CD28 mAbs (both at 0.5 μg/ml; BD Pharmingen) in the presence of human IL-2 (100 U/ml; BD Pharmingen). IL-12+ splenocytes were stimulated with 1.0 μg/ml OVA257–264 peptide, Recombinant murine IL-12 (5 ng/ml; PeproTech) and the neutralizing mAb anti-IFN-γ (hybridoma clone XMG-6; 40 μg/ml) were added to the indicated wells.

Bone marrow chimeras

B6.Ly5.1/Cr mice were sublethally irradiated and partially reconstituted with bone marrow from IL-12Rβ2−/− mice. After 60 days, the animals

---

**FIGURE 1.** More memory CD8 T cells in $p35^{-/-}$ mice after *L. monocytogenes* infection. WT and $p35^{-/-}$ mice were immunized with rLmOVA and OVA-specific cells were analyzed 7 days or 60 days postinfection by intracellular IFN-γ and Kb/OVA tetramer staining (A and B). A, Dot plots show IFN-γ-producing splenic CD8 T cells (numbers indicate percentage of CD8 T cells that produce IFN-γ). Bar graphs represent the total number of Kb/OVA tetramer+ CD8 T cells per spleen (means ± SD). B, Bar graph shows the percentage of TE over TM cells (number of TM cells on day 7). C, Dot plots show OVA-specific memory CD8 T cells in the liver, spleen, lymph node, and bone marrow 60 days postinfection. D, WT and $p35^{-/-}$ mice were immunized with $5 \times 10^5$ CFU of rLm for naive mice (left panel, primary) and $5 \times 10^6$ CFU of rLm for immune mice (right panel, challenge). Bar graphs show CFU per g of liver 2 days postimmunization (means ± SD). E, WT and $p35^{-/-}$ mice were immunized with rLmOVA and challenged 60 days later with rLmOVA. OVA-specific cells in the spleens of challenged mice 8 days after secondary immunization ($2^\text{nd} \text{TE}$) and unchallenged mice (TM) were detected by intracellular cytokine staining. Numbers represent the percentage of CD8 T cells that were IFN-γ+. The bar graphs show the numbers of OVA-specific CD8 T cells per spleen (means ± SD). F and G, Equal numbers of OVA-specific CD8 T cells from WT and $p35^{-/-}$ mice (Thy1.2+; purple dots) were injected into WT recipients (Thy1.1+; blue dots) that were then challenged with rLmOVA. Splenocytes were restimulated with the OVA peptide, and intracellular IFN-γ was measured 3 days postchallenge (F) and bacterial clearance was measured in the spleen and liver (G). Bar graphs represent the number of Kb/OVA+ CD8 T cells (F) or CFU per organ (G) (means ± SD).
were bled and T cells were analyzed by surface staining and flow cytometry to confirm chimera. Mice were then immunized with rLmOVA and analyzed 7 days or 60 days after immunization.

Adoptive transfer

Splenocytes from WT and p35\(^{-/-}\) mice were stained with K\(^{b}\)/OVA tetramer to determine numbers of OVA-specific cells, and splenocytes containing \(1 \times 10^9\) OVA-specific cells were transferred i.v. to recipient mice followed by challenge infection as indicated.

Results

Greater CD8 T\(_M\) in p35\(^{-/-}\) mice after rLmOVA immunization

During the course of our studies to investigate the ability of Th1 cells to assist CD8 T\(_M\) development, we unexpectedly discovered that IL-12-deficient mice had substantially more CD8 T\(_M\) cells despite a weaker Th\(_1\) response after immunization with rLmOVA. The numbers of OVA-specific CD8 T\(_E\) cells in p35\(^{-/-}\) mice 7 days after rLmOVA immunization were roughly one-half of those in WT mice, while 60 days postimmunization there were 3-fold or 1.4 \(\times 10^6\) more OVA-specific CD8 T\(_M\) cells in p35\(^{-/-}\) mice compared with WT (Fig. 1A). This increase in OVA-specific CD8 T\(_M\) cells was consistently observed when calculated as percentages of CD8 T cells or as absolute numbers per spleen and when measured by tetramers or intracellular IFN-\(\gamma\) staining. The difference in CD8 T\(_M\) is particularly striking in light of the weaker primary response in p35\(^{-/-}\) animals. Although the number of CD8 T\(_M\) cells was 3% of OVA-specific CD8 T\(_E\) cells in WT mice, the number of CD8 T\(_M\) cells was >30% of OVA-specific CD8 T\(_E\) cells in p35\(^{-/-}\) mice (Fig. 1B). The increase in CD8 T\(_M\) formation was also observed in the lymph nodes, liver, and bone marrow (Fig. 1C) and in IL-12p40\(^{-/-}\) mice (data not shown). Taken together, our results clearly show that although IL-12 promotes the CD8 T\(_E\) cell response, there is enhanced generation of CD8 T\(_M\) in the absence of IL-12.

IL-12-deficient mice are more susceptible to primary infection, but more resistant to reinfection

Because p35\(^{-/-}\) mice have greater CD8 T\(_M\) cells, we investigated whether they were more protected from reinfection. We measured bacterial numbers in naive and immune WT and p35\(^{-/-}\) mice 2 days after infection with rLmOVA. As expected, naive p35\(^{-/-}\) mice were more susceptible and had 1 log more bacteria than WT mice after primary infection (Fig. 1D, left panel). However, immune p35\(^{-/-}\) mice had 3 logs fewer bacteria compared with immune WT mice after challenge infection (Fig. 1D, right panel). Thus, although IL-12-deficient mice were more susceptible to primary infection, they became more resistant to reinfection. Increased resistance of IL-12-deficient mice to reinfection correlated with a robust recall response in p35\(^{-/-}\) mice: 8 days postchallenge, \(\sim 19\%\) of CD8 T cells in p35\(^{-/-}\) mice were OVA specific compared with only 8% in WT mice (Fig. 1E). These results show that a stronger secondary T\(_E\) response is generated as a result of more T\(_M\) cells in p35\(^{-/-}\) mice that confer greater protective immunity to reinfection.

To compare the functionality of CD8 T\(_M\) on a per cell basis, we adoptively transferred equal numbers of OVA-specific T\(_E\) cells from immune WT and p35\(^{-/-}\) mice into naive congenic WT recipients. By day 3 postchallenge with rLmOVA, there were more OVA-specific secondary T\(_E\) derived from p35\(^{-/-}\) donor T\(_M\) cells than from WT donor T\(_M\) cells (Fig. 1F). Mice that received p35\(^{-/-}\) donor T\(_M\) had 2 logs fewer bacteria compared with the mice that received WT donor T\(_M\) (Fig. 1G). These results demonstrate that CD8 T\(_M\) cells from IL-12-deficient mice are fully capable of mounting enhanced recall responses that mediate greater protective immunity to reinfection.

More CD8 T\(_M\) in rLmOVA-immunized mice

IL-12-deficient mice have greater CD8 T\(_M\) cells than WT mice (Fig. 2A). WT and p35\(^{-/-}\) mice were immunized with a highly attenuated strain of rLmOVA (ΔactA rLmOVA), which renders the bacteria unable to spread from cell to cell in the host. Bacterial clearance was measured in the spleen 2 and 4 days postimmunization. Bar graphs show the CFU per spleen (means \(\pm\) SD). B, WT and p35\(^{-/-}\) mice were immunized with ΔactA rLmOVA treated with 2 mg/ml ampicillin in their drinking water starting 14 days postinfection until sacrificed 7 days postinfection. Splenocytes were stained with K\(^{b}\)/OVA tetramer or restimulated with OVA peptide and intracellular IFN-\(\gamma\) was measured (CD8\(^{+}\) T cell gated). Numbers indicate the percentage of CD8 T cells. Bar graphs represent the number of IFN-\(\gamma\)-positive CD8 T cells per spleen (means \(\pm\) SD).

More CD8 T\(_M\) in rLmOVA-immunized mice

IL-12-deficient mice are more susceptible to rLm infection although they are capable of clearing a sublethal infection (28). Although p35\(^{-/-}\) mice had higher bacterial loads at day 3 (Fig. 2D, left panels), both WT and p35\(^{-/-}\) mice cleared infection by 7 days after rLmOVA immunization (data not shown). To examine whether enhanced CD8 T\(_M\) in p35\(^{-/-}\) mice is due to differences in bacterial load and Ag stimulation, we immunized WT and p35\(^{-/-}\) mice with a highly attenuated strain of rLmOVA (ΔactA rLmOVA) that is unable to spread from cell to cell and is quickly cleared in both WT and p35\(^{-/-}\) mice. On day 2 postinfection, there were comparable levels of bacteria in the spleens and livers between WT and p35\(^{-/-}\) mice (Fig. 2A). By day 4 postinfection, both WT and p35\(^{-/-}\) mice cleared the infection in the spleen and liver and no bacteria were detected in other organs at any time points (Fig. 2A). To further eliminate any possible persisting bacteria, we treated ΔactA rLmOVA-immunized WT and p35\(^{-/-}\) mice with antibiotics and measured OVA-specific CD8 T\(_M\) 60 days later. More CD8 T\(_M\) cells were still present in p35\(^{-/-}\) mice than in WT (Fig. 2B). Together, these results show that greater CD8 T\(_M\) in p35\(^{-/-}\) mice is not due to differences in the level or duration of Ag stimulation during infection.

More CD8 T\(_M\) in IL-12-deficient mice is not simply due to reduced contraction

A recent study by Badovinac et al. (6) has shown that without inflammation there is reduced contraction of CD8 T cell populations following infection. Another study by Van Faasen et al. (29)
and 3 wk after infection, we performed kinetic analyses of OVA-specific CD8 T cells in WT and p35−/− mice. WT and p35−/− mice were immunized with ΔactA rLmOVA and OVA-specific cells analyzed in the spleen on days 7, 14, and 21 after infection (A–G). A. Dot plots show splenocytes stained with Kb/OVA tetramer (Kb/OVA+ gate). Numbers indicate the percentage of CD8 T cells that are Kb/OVA+. Line graphs represent the (B) percentage of CD8 T cells that were Kb/OVA+ (means ± SE) or (C) the total number of Kb/OVA+ cells per spleen (means ± SD). D. Histograms show surface expression of IL-7Rα (Kb/OVA+ gated for days 7–21 and isotype control; total CD8+ gated for naive mouse; WT, no fill; p35−/−, shaded). The ability of OVA-specific CD8 T cells to produce IL-2 was determined by intracellular cytokine staining (E–G). E. Dots plots show CD8+ gated cells; numbers indicate the percentage of IFN-γ+ cells that produced IL-2. Line graphs represent the percentage (means ± SD) (F) or the number (means ± SE) (G) of IFN-γ+ cells that produced IL-2. More CD8 TM precursors are generated in p35−/− mice. WT and p35−/− mice were immunized with ΔactA rLmOVA immunization to more carefully demonstrate the role of T cell stimulation, governed by inflammation, in negatively influencing commitment to TM cells. Because IL-12 is a proinflammatory cytokine, we considered the possibility of reduced contraction in p35−/− mice leading to more CD8 TM. Because the majority of Tc1 cell death occurs between 2 and 3 wk after infection, we performed kinetic analyses of OVA-specific CD8 T cells in WT and p35−/− mice from days 7 to 21 following ΔactA rLmOVA immunization to more carefully study the amount of actual Tc1 cell contraction that occurs in p35−/− mice.

At day 7 following immunization, the magnitude of CD8 Tc1 cell expansion in p35−/− mice was lower than in WT mice (Fig. 3, A–C), similar to the results we observed following rMOVA infection (Fig. 1A). This is consistent with published findings showing that IL-12 is important for CD8 Tc1 cell responses (17–19). Like in IFN-γ−/− mice (30), there is a reduced contraction of Ag-specific CD8 T cells in p35−/− mice. The fold contraction in WT animals is ∼9- to 10-fold, whereas it is ∼3- to 4-fold in p35−/− mice, as determined by comparing the numbers of OVA-specific CD8 T cells at days 7 and 21. Although the fold contraction was less in p35−/− mice, the numbers of OVA-specific cells in WT and p35−/− mice were similar at day 21 (1.2% and 1.2% of CD8 T cells or 1.4 × 105 and 1.5 × 105 cells/spleen, respectively; Fig. 3, A–C). Thus, at the end of the contraction phase, WT and p35−/− mice had similar numbers of total OVA-specific CD8 T cells, and the greater number of long-term TM cells in p35−/− mice is not simply due to reduced contraction.

More CD8 Tm precursors in p35−/− mice

Ag-specific CD8 T cells responding to infection are not a homogeneous population of effectors, but consist of a small number of memory precursors, which express IL-7Rα and IL-2 and gradually differentiate into long-term TM (5). To examine whether more TM precursors were generated in the absence of IL-12, we measured expression of IL-7Rα and IL-2 in OVA-specific cells following ΔactA rLmOVA immunization. Greater percentages of OVA-specific CD8 T cells in p35−/− mice expressed high levels of IL-7Rα and IL-2 compared with WT mice (Fig. 3, D–F). These differences were observed as early as day 7 at the peak of the primary response and continued throughout the contraction phase. Although the overall number of OVA-specific cells was less in p35−/− mice at day 7 (Figs. 1A and 3C), there were greater numbers of IL-2-producing OVA-specific TM precursors in p35−/− mice compared with WT mice (Fig. 3G). As the total number of OVA-specific cells contracted to a similar level by day 21, the number of IL-2-producing OVA-specific TM precursors remained higher in p35−/− than in WT mice. These data suggest that during the primary response more OVA-specific TM precursors are generated in IL-12-deficient animals, thus leading to greater OVA-specific CD8 Tm.

IL-7Rα and IL-2 expression by Ag-specific CD8 T cells correlate with the ability of these cells to undergo proliferative renewal. This proliferative process is critical for maturation of TM precursors into fully differentiated TM cells and their long-term maintenance (2, 4, 5, 9, 31). We examined the proliferation of OVA-specific CD8 Tm cells in rLmOVA-immunized p35−/− and WT
animals by administering BrdU. We found that 39% of OVA-specific CD8 T cells in p35−/− mice had incorporated BrdU compared to only 14% in WT mice. This enhanced proliferation in p35−/− mice was only observed for Ag-specific CD8 T\textsubscript{M} cells, while the total CD8 T cells incorporated BrdU at the same rate between the groups (Fig. 3H). Thus, there is either enhanced proliferation of Ag-specific CD8 cells or more of them undergoing proliferative renewal in p35−/− mice than in WT mice. These results further support our finding that more long-term CD8 T\textsubscript{M} cells are generated and maintained in p35−/− mice.

**IL-12 regulation of CD8 T\textsubscript{M} development is dependent on signaling via the IL-12R on CD8 T cells**

IL-12 could suppress CD8 T\textsubscript{M} either by signaling through its receptor on CD8 T cells or by signaling through its receptor on an intermediate cell. For example, IL-12 can activate NK cells to produce large amounts of IFN-γ (32) that might influence CD8 T\textsubscript{M} development. To distinguish these two possibilities, we generated IL-12R\textsubscript{2} bone marrow chimeras, which have one population of T cells expressing the IL-12R (Ly5.1\textsuperscript{+}) and another population lacking a functional IL-12R (Ly5.2\textsuperscript{+}), thus allowing a direct comparison of these two populations within the same animal. On day 7 after rLmOVA immunization, OVA-specific CD8 T cells were comprised equally of IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} and IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} populations (Fig. 4, A and B). By day 60 >80% of the OVA-specific T\textsubscript{M} cells were now of IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} origin (Fig. 4, A and B). Furthermore, greater numbers of OVA-specific IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} cells expressed high levels of IL-7R\textsubscript{a} and produced IL-2 compared with IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} cells (Fig. 4, C and D). These results show that IL-12 signaling via its cognate receptor on CD8 T cells regulates the generation of CD8 T\textsubscript{M} precursors and their differentiation into long-lasting T\textsubscript{M}.

**IL-12 enhances activation of CD8 T cells and effector functions while suppressing IL-2 and IL-7R expression**

Our in vivo results suggest that IL-12 enhances the generation of fully activated CD8 T\textsubscript{E} cells while inhibiting the formation of CD8 T\textsubscript{M} precursors (expressing IL-7R\textsubscript{a} and IL-2) during a primary response, leading to reduced long-term CD8 T\textsubscript{M}. To examine the mechanism by which IL-12 regulates CD8 T\textsubscript{M} development, we took a reductionist approach and stimulated OT-I cells in vitro in the presence or absence of exogenous IL-12. OT-I cells cultured with IL-12 produced more IFN-γ (compare mean fluorescence intensity of 131 and 98 with and without IL-12, respectively). However, fewer OT-I cells produced IL-2 or expressed IL-7R\textsubscript{a} when cultured with exogenous IL-12 (Fig. 5A). These data recapitulate our in vivo results and clearly show that IL-12 enhances the effector function of IFN-γ production, while suppressing the expression of IL-2 and IL-7R\textsubscript{a} that are conducive for T\textsubscript{M} development.

Activation increases the amount of ROS in T cells and these molecules have been shown to modulate the apoptosis/survival of activated T cells (33–36). We tested whether IL-12-driven T\textsubscript{E} produced more ROS because IL-12 enhances T cell activation and the expression of effector functions. OT-I cells stimulated in vitro with OVA peptide and exogenous IL-12 produced substantially more ROS (43% compared 18% without IL-12; Fig. 5B). Consistent with this in vitro finding, more CD8 T cells from L. monocytogenes-infected WT mice expressed high levels of ROS compared to CD8 T cells from infected p35−/− mice (Fig. 5C). These data indicate that IL-12 drives greater ROS production in CD8 T\textsubscript{E} and suggest that IL-12-driven CD8 T\textsubscript{E} are more likely to undergo apoptosis and thus less likely to differentiate into T\textsubscript{M} cells.

To ascertain a direct role of IL-12, we examined whether signaling directly through its cognate receptor on CD8 T cells was required or whether it involved intermediates such as IFN-γ, which is highly induced by IL-12. Splenocytes from IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} and IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} bone marrow chimera mice were analyzed 7 and 60 days after immunization with rLmOVA. A, OVA-specific CD8 T cells were detected by K\textsubscript{o}OVA tetramer (left panels, numbers indicate the percentage of CD8 T cells that are K\textsubscript{o}OVA\textsuperscript{+}) and the composition of this population was assessed for IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} (Ly5.1\textsuperscript{+}) or IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} (Ly5.2\textsuperscript{+}) origin (right panels, K\textsubscript{o}OVA\textsuperscript{+} gated). B, OVA-specific CD8 T cells were detected by intracellular IFN-γ staining and the composition of this population was assessed for IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} (Ly5.1\textsuperscript{+}) or IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} (Ly5.2\textsuperscript{+}) origin (gated on IFN-γ\textsuperscript{+} cells). C, IL-7R\textsubscript{a} expression on OVA-specific IL-12R\textsubscript{2}/Ly5.2\textsuperscript{+} cells (no fill, K\textsubscript{o}OVA\textsuperscript{+} Ly5.1\textsuperscript{+} gated) vs IL-12R\textsubscript{2}/Ly5.1\textsuperscript{+} cells (shaded, K\textsubscript{o}OVA\textsuperscript{+} Ly5.2\textsuperscript{+} gated). Numbers indicate the percentage of K\textsubscript{o}OVA\textsuperscript{+} cells that express high levels of IL-7R\textsubscript{a}. D, The ability of OVA-specific CD8 T cells to produce IFN-γ and IL-2 were measured by intracellular cytokine staining (CD8\textsuperscript{+} gated; numbers indicate percentage of IFN-γ\textsuperscript{+} cells that are IL-2\textsuperscript{+}).
Prime boost immunization in the absence of IL-12 induces more \( T_M \) precursors and long-lasting \( T_M \)

Because prime boost immunization is a common vaccination protocol to induce long-lived CD8 \( T_M \), we examined the effects of IL-12 on the efficacy of prime boost immunization. WT and \( p35^{-/-} \) mice were immunized with rLmOVA. On day 7 following the boost, 70% of OVA-specific CD8 \( T_M \) in \( p35^{-/-} \) mice expressed high levels of IL-7R\( \alpha \) compared with only 30% in WT mice (Fig. 6A). Thus, the difference in IL-7R\( \alpha \) expression between \( p35^{-/-} \) and WT mice seen in primary \( T_E \) became strikingly more apparent in secondary \( T_E \). These data further support the conclusion that IL-12 regulates the generation of CD8 \( T_M \) precursors by modulating IL-7R\( \alpha \) expression, and suggest that prime boost immunization in the absence of IL-12 would induce more long-lasting CD8 \( T_M \) cells. Indeed, when we measured OVA-specific CD8 \( T_M \) cells 60 days after boost, the \( p35^{-/-} \) mice maintained a larger population that expressed high levels of IL-7R\( \alpha \) as compared with only 30% in WT mice (Fig. 6B). These results show that the absence of IL-12, substantially more CD8 \( T_M \) precursors are generated among secondary \( T_E \) following prime boost immunization, leading to greater numbers of long-lived CD8 \( T_M \) cells.

Discussion

Most studies have focused on the roles of Ag dose and persistence in regulating CD8 T cell expansion and \( T_M \) differentiation (37, 38). In this study, we describe the novel finding that the cytokine IL-12 has the opposing properties of promoting the \( T_E \) cell response while inhibiting the development of CD8 \( T_M \). We show that IL-12-deficient mice have a reduced CD8 \( T_E \) cell expansion and contraction following \( L. monocytogenes \) immunization, but a gradual increase in the numbers of CD8 \( T_M \) cells formed due to enhanced homeostatic renewal.

To examine the mechanism by which IL-12 regulates CD8 \( T_M \), we first considered whether increased CD8 \( T_M \) in \( p35^{-/-} \) mice resulted from altered infection. Our results with the attenuated mutant \( \Delta actA \) rLmOVA, which is cleared at similar rates in WT and \( p35^{-/-} \) mice, as well as those with the antibiotic treatment indicate that differences in the level or duration of Ag stimulation following infection do not account for greater CD8 \( T_M \) in the absence of IL-12. Furthermore, our studies of IL-12 bone marrow chimera conclusively rule out this possibility because experiments were performed within the same chimeric mouse, where the rate of bacterial clearance is not an issue, and still more IL-12R\( \beta^2^{-/-} \) than IL-12R\( \beta^2^{+/+} \) cells developed into CD8 \( T_M \) cells. These results also demonstrate that IL-12 regulation of CD8 \( T_M \) depends on direct signaling via its receptor on CD8 T cells, rather than through intermediate factors such as IFN-\( \gamma \). This is further supported by our in vitro studies using cocultures of IL-12R\( \beta^2^{-/-} \) and IL-12R\( \beta^2^{+/+} \) cells with IFN-\( \gamma \) neutralization (Fig. 5).

Previous studies have shown reduced CD8 T cell contraction in the absence of inflammation (6). We examined whether this was the mechanism underlying increased CD8 \( T_M \) in \( p35^{-/-} \) mice. Our results show that the total number of Ag-specific CD8 T cells contract to a similar level in \( p35^{-/-} \) and WT mice. However, the quality of the Ag-specific CD8 T cell populations in WT and \( p35^{-/-} \) mice begins to diverge early in the response in terms of the percentage of cells that express \( T_E \) or \( T_M \) phenotypes (Fig. 3, B–E). This divergence in quality resolves the conundrum of why the overall numbers of Ag-specific cells during the contraction phase in the WT and \( p35^{-/-} \) mice are similar (Fig. 3A), while the latter animals go on to have greater numbers of \( T_M \) cells.

Why are more \( T_M \) precursors generated in \( p35^{-/-} \) mice? In accordance with the signal strength model (39), CD8 T cells are driven less vigorously to become \( T_M \) in the absence of IL-12, and thus are more likely to become \( T_M \) precursors that develop into

### FIGURE 5

IL-12 suppresses IL-2 and IL-7R\( \alpha \) expression while enhancing ROS production in CD8 T cells. A and B, OT-I splenocytes were stimulated with OVA peptide in the presence or absence of exogenous IL-12 for 3 days. A, Dot plots show IFN-\( \gamma \)-producing CD8 T cells. The numbers indicate the mean fluorescent intensity of IFN-\( \gamma \) for IFN-\( \gamma \)-producing cells (top panels), percentage of IFN-\( \gamma \)-producing cells that produce IL-2 (middle panels), and the percentage of IL-2-producing cells that express high levels of IL-7R\( \alpha \) (IFN-\( \gamma \)-gated; bottom panels). B, Histogram shows ROS production and numbers indicate the percentage of CD8 T cells that produce high levels of ROS (CDM\( ^{+/-} \)-gated; +IL-12, shaded; no IL-12, unshaded). C, WT and \( p35^{-/-} \) mice were immunized with rLm and ROS was measured in splenocytes ex vivo 2 days later. Histograms show ROS production by CD8 T cells from WT (solid line), \( p35^{-/-} \) (dashed line), and the negative control (shaded area). D and E, Equal numbers of IL-12R\( \beta^2^{+/+} \) and IL-12R\( \beta^2^{-/-} \) splenocytes were mixed and stimulated with anti-CD3/CD28 with or without exogenous IL-12. D, Histograms show IL-2 production by activated CD8 T cells (IL-12R\( \beta^2^{+/+} \), no fill; IL-12R\( \beta^2^{-/-} \), shaded). The numbers represent the percentage of activated CD8 T cells that produce IL-2. E, ROS production was measured in CD8 T cells cultured in the presence of IL-12 and/or anti-IFN-\( \gamma \). Numbers within the histograms represent the percentage of CD8 T cells that produce high levels of ROS.

### FIGURE 6

Generation of more CD8 \( T_M \) precursors and long-lasting \( T_M \) cells following prime boost immunization in the absence of IL-12. A, WT (no fill) and \( p35^{-/-} \) (shaded) mice were primed with rLmOVA and one group was boosted with rLmOVA 60 days after the first immunization. Histograms (K\( ^{35} \)OVA\( ^{+/-} \)gated) show surface expression of CD62L and IL-7R\( \alpha \) on OVA-specific \( T_M \) (60 days after prime) and secondary \( T_E \) (8 days after boost). B, WT and \( p35^{-/-} \) mice were primed and then boosted 60 days later with rLmOVA. Two months after boost, OVA-specific CD8 \( T_M \) cells were detected by K\( ^{35} \)OVA tetramer (top panels; CD\( ^{+/-} \) gated) and their expression of CD62L and IL-7R\( \alpha \) was examined (bottom panels; K\( ^{35} \)OVA\( ^{+/-} \) gated).
long-term Tₘ. Our in vitro studies further show that IL-12 acts on CD8 T cells to enhance effector characteristics such as IFN-γ and ROS production, while inhibiting their expression of IL-2 and IL-7Rα. In addition to inducing IFN-γ and ROS production, it is likely that IL-12 up regulates a panel of genes that are key for inducing Tₘβ, just as it down-regulates another set of genes in addition to IL-2 and IL-7Rα, which are critical for Tₘγ generation. Together, these results provide a mechanistic explanation for how IL-12 has the opposite effect of promoting a primary T cell response while inhibiting Tₘ development. By signaling directly through its receptor on CD8 T cells, IL-12 regulates gene expression and influences CD8 T cell differentiation in a way that favors the generation of fully activated TₘC, while hindering the formation of CD8 Tₘγ/δ precursors and differentiation of long-term CD8 Tₘ. A recent study (40) has shown that IL-12 represses the expression of the transcription factor Eomesodermin in Ag-specific CD8 T cells during infection. In mice previously been shown to play an important role in controlling CD8 T cell function and fate (41, 42).

Our findings suggest a paradigm in which the early innate response not only promotes the adaptive Tₘβ response, but also regulates Tₘγ differentiation. This makes biological sense in the context of an infection. When dendritic cells produce IL-12 in response to microbial stimulation, they ensure maximal IFN-γ production and TₘC cell activation, which are important for pathogen clearance. The adverse effect of IL-12 on Tₘ development might ensure that the diversion of Ag-specific cells away from the Tₘ pool into the Tₘ pool occurs only after the infection is controlled and inflammation has subsided, indicating “no more danger” (43). Consistent with this model, a recent study by Williams and Bevan (44) has suggested that the stimuli received during the early stages of infection promote the generation of Tₘβ, whereas cues at the later stages of infection influence Tₘγ differentiation. Our data indicate that IL-12 provides a key signal through which the innate response orchestrates the subsequent adaptive response to ensure the induction of potent TₘC that first clear the pathogen, followed by the generation of long-lasting Tₘγ that mediates protective immunity against reinfection.

One of the most surprising aspects of this study was the observation that p35−/− mice are more resistant to reinfection while they are more susceptible to primary infection. The fact that the p35−/− mice have a smaller primary Tₘβ response, but more Tₘγ clearly underlies this difference in susceptibility and illustrates the important point that a large primary response does not de facto dictate a large population of long-lived Tₘγ cells. This finding is of significance for prophylactic vaccine development, because it illustrates that: 1) a priming immunization need not induce a large Tₘβ response and 2) IL-12 and other proinflammatory adjuvants may not be the most favorable choices for inducing long-lived CD8 Tₘ. Although IL-12 helps therapeutic vaccines that aim to drive large Tₘβ responses (23), the use of IL-12 as an adjuvant in prophylactic vaccines may not achieve its intended goal of inducing a large population of long-lived Tₘγ cells. Instead, our results suggest that new strategies exploiting the opposite roles of IL-12 on the primary response and memory formation will achieve an optimal effect for enhancing vaccine efficacy.

Acknowledgments
We thank Kathy Foulds, Jiu Jiang, Amy Troy, John Northrop, Connie Krawczyk, Rusty Jones, Jie Sun, and Edward Pearce for discussion, advice, and assistance.

Disclosures
The authors have no financial conflict of interest.

References
6. Lee, J. R., and G. A. Koretzky. 1998. Production of reactive oxygen intermediates (ROS) during Ag-specific CD8 T cell responses to microbial stimulation, they ensure maximal IFN-γ production and TₘC cell activation, which are important for pathogen clearance. The adverse effect of IL-12 on Tₘ development might ensure that the diversion of Ag-specific cells away from the Tₘ pool into the Tₘ pool occurs only after the infection is controlled and inflammation has subsided, indicating “no more danger” (43). Consistent with this model, a recent study by Williams and Bevan (44) has suggested that the stimuli received during the early stages of infection promote the generation of Tₘβ, whereas cues at the later stages of infection influence Tₘγ differentiation. Our data indicate that IL-12 provides a key signal through which the innate response orchestrates the subsequent adaptive response to ensure the induction of potent TₘC that first clear the pathogen, followed by the generation of long-lasting Tₘγ that mediates protective immunity against reinfection.

One of the most surprising aspects of this study was the observation that p35−/− mice are more resistant to reinfection while they are more susceptible to primary infection. The fact that the p35−/− mice have a smaller primary Tₘβ response, but more Tₘγ clearly underlies this difference in susceptibility and illustrates the important point that a large primary response does not de facto dictate a large population of long-lived Tₘγ cells. This finding is of significance for prophylactic vaccine development, because it illustrates that: 1) a priming immunization need not induce a large Tₘβ response and 2) IL-12 and other proinflammatory adjuvants may not be the most favorable choices for inducing long-lived CD8 Tₘ. Although IL-12 helps therapeutic vaccines that aim to drive large Tₘβ responses (23), the use of IL-12 as an adjuvant in prophylactic vaccines may not achieve its intended goal of inducing a large population of long-lived Tₘγ cells. Instead, our results suggest that new strategies exploiting the opposite roles of IL-12 on the primary response and memory formation will achieve an optimal effect for enhancing vaccine efficacy.

Acknowledgments
We thank Kathy Foulds, Jiu Jiang, Amy Troy, John Northrop, Connie Krawczyk, Rusty Jones, Jie Sun, and Edward Pearce for discussion, advice, and assistance.

Disclosures
The authors have no financial conflict of interest.

References