CD4+ T cell help and innate-derived IL-27 induce Blimp-1-dependent IL-10 production by antiviral CTLs

Jie Sun1,2, Haley Dodd1, Emily K Moser1, Rahul Sharma3 & Thomas J Braciale1,2,4

Interleukin (IL)-10 is an important regulatory cytokine that can modulate excessive immune mediated injury. Several distinct cell types have been demonstrated to produce IL-10, including most recently CD8+ cytotoxic T lymphocytes (CTLs) responding to respiratory virus infection. Here we report that CD4+ T cell help in the form of IL-2 is required for IL-10 production by CTLs, but not for the induction of CTL effector cytokines. We show that IL-2 derived from CD4+ helper T cells cooperates with innate immune cell–derived IL-27 to amplify IL-10 production by CTLs through a Blimp-1-dependent mechanism. These findings reveal a previously unrecognized pathway that coordinates signals derived from innate and helper T cells to control the production of a regulatory cytokine by CTLs during acute viral infection.

CD4+ T cells are a chief producer of IL-10 in the respiratory tract during influenza infection6. CTL-derived IL-10 is critical for preventing excess inflammation during immune-mediated virus clearance6. In this report, we investigate the signals necessary to drive the production of IL-10 by CTLs in the infected respiratory tract. Our data identify an interplay between products of helper T cells and infiltrating innate immune cells in shaping the function of antiviral CTLs at the site of infection.

RESULTS

IL-10+ CTL development requires IL-27 and helper T cells

To investigate the mechanisms regulating IL-10 production by antiviral CTLs during influenza infection, we first examined whether interferon-γ (IFN-γ) is required, as the IL-10-producing CTLs coexpress IFN-γ (Supplementary Fig. 1). IFN-γ was dispensable for the induction of IL-10-producing CTLs (Supplementary Fig. 1). Similarly, although IL-12 is important for inducing IL-10-producing T cells in some settings17, it was not required for the induction of IL-10-producing CTLs during influenza infection, as CD8+ T cells from Il12a−/− mice, null for the p35 subunit of the cytokines IL-12 and IL-35 (ref. 18) could produce IL-10 (Fig. 1a). IL-27 induces the development of IL-10-producing CD4+ T cells3; therefore, we examined the role of IL-27 in controlling the induction of IL-10-producing CTLs during influenza infection. Influenza infection induced IL-27 gene expression in the respiratory tract (Supplementary Fig. 2). We then infected IL-27 subunit–deficient Ebi3−/− mice (which lack both IL-27 and IL-35; ref. 18) with influenza and measured IL-10 production by influenza-specific CTLs. EBI-3 deficiency did not affect the induction of IFN-γ-producing CTLs, but it substantially impaired the induction of IL-10-producing CTLs in both draining lymph nodes (the mediastinal lymph nodes, MLNs) and the infected lungs (Fig. 1b,c). Consistent with the impaired production of IL-10 by CTLs, Ebi3−/− mice showed impaired IL-10 release in the airway and enhanced pulmonary inflammation during influenza infection (Supplementary Fig. 3). The induction of IL-10-producing CD4+ T cells by IL-27 depends on IL-21 signaling10.
however, the induction of IL-10–producing CTLs during influenza infection was intact in mice lacking IL-21 receptor (Fig. 1d).

The absence of IL-27 did not completely abrogate IL-10–producing CTLs, suggesting that other signals contribute to the induction of these cells. Release of IL-10 in vivo depends on both CD8+ and CD4+ T cells, suggesting a possible role of CD4+ T cells in the induction of IL-10–producing CTLs. We thus eliminated both IL-27 and CD4+ T cells in vivo and examined the induction of IL-10–producing CTLs upon influenza infection. Depletion of CD4+ T cells, together with EBI-3 deficiency, almost completely abrogated the induction of IL-10–producing CTLs (Fig. 1e, f). Elimination of CD4+ T cells and IL-27 also compromised the production of IL-10 at the single-cell level (Fig. 1g) in the few remaining IL-10+ CTLs (Fig. 1g). Taken together, these data suggest that the induction of IL-10–producing CTLs during influenza infection depends on the presence of both IL-27 and helper T cells, but is independent of IL-12, IL-21, IL-35 and IFN-γ.

Helper T cells selectively induce IL-10+ CTL development

The finding that the induction of IL-10–producing CTLs in vivo required the presence of CD4+ T cells raised the possibility that CD4+ T cell ‘help’ is required for physiologic CTL responses to influenza infection. We therefore examined the role of CD4+ T cells in the accumulation and differentiation of CTLs in the respiratory tract during influenza infection. The depletion of CD4+ T cells severely impaired the production of IL-10, but not IFN-γ or tumor necrosis factor (TNF), by CTLs (Fig. 2a, b). These data agree with reported findings and suggest that CD4+ T cell help is minimally required for the differentiation and accumulation of CTLs. CD4+ T cell depletion at various time points impaired the development of IL-10–producing CTLs, even at 6 days after infection (d.p.i.)—that is, when T cells have entered the infected lungs—suggesting that CD4+ T cells are able to help CD8+ T cells in the lung as well as in the MLNs (Supplementary Fig. 4).

To further confirm the effect of CD4+ T cell depletion, we infected H2-Ab1−/− (major histocompatibility complex (MHC) type II) mice, which lack CD4+ T cells, with influenza and found that CD8+ T cells from H2-Ab1−/− mice were likewise impaired in IL-10 but not IFN-γ or TNF production (Fig. 2c). The absence of CD4+ T cells only minimally affected the expression of the cytolytic molecule granzyme B by CTLs (Supplementary Fig. 5). We next directly examined the role of CD4+ T cell help in the in vivo induction of IL-10–producing CTLs by using IL-10 reporter (Vert-X) mice. IL10 gene expression in CTLs was primarily restricted to the site of infection, the lungs (Supplementary Fig. 6). Notably, the depletion of CD4+ T cells significantly impaired the development of IL-10–expressing cells among both total lung-infiltrating CD8+ T cells and gated influenza antigen–specific cells (Fig. 2d and Supplementary Fig. 6). We also bred Vert-X mice to H2-Ab1−/− mice and infected Vert-X H2-Ab1−/− mice with influenza. Compared to the influenza–specific CD8+ T cells from littermate Vert-X H2-Ab1−/− mice, influenza–specific T cells from Vert-X H2-Ab1−/− mice had diminished IL-10–expressing cell numbers (Fig. 2e) and IL-10 expression intensity at the single-cell level (Supplementary Fig. 7). We also verified in vivo that CD4+ T cell help was not required for the induction of IFN-γ–expressing cells using IFN-γ–enhanced yellow fluorescent protein (eYFP) reporter (Yeti) mice, which were constructed similarly to the Vert-X mice (Fig. 2f and Supplementary Fig. 8). Collectively, these data suggest that CD4+ T cell help is critical for inducing an optimal anti-inflammatory cytokine profile (that is, IL-10 production) but not for the activation and differentiation of the effector features of influenza–specific CTLs in vivo.

IL-2 provides help for IL-10+ CTL development

To identify the molecule(s) that mediate the help from CD4+ T cells, we developed an in vitro T cell–dendritic cell (DC) culture system. We labeled influenza hemagglutinin–specific CD8+ T cell receptor (TCR) transgenic T cells (CL-4) with the cytosolic dye CFSE and
CD4⁺ T cell help is selectively required for the induction of IL-10-producing CTLs in vivo. (a,b) Production of IL-10, IFN-γ and TNF (a) by CTLs from MLNs or lungs of WT mice injected with rat immunoglobulin G (IgG) or anti-CD4 to deplete CD4⁺ T cells and infected with influenza, measured 8 d.p.i. by ICS. Numbers are the percentages of cells in gated population. (b) Normalized percentages of IL-10⁺ or TNF⁺ cells in influenza-specific CTLs (IFN-γ⁺) from lungs of WT and H2-AbI−/− mice infected with influenza, 7 d.p.i. (c,d) Normalized percentages of IL-10⁺ or TNF⁺ cells in influenza-specific CTLs (IFN-γ⁺) from lungs of WT and H2-AbI−/− mice infected with influenza, 7 d.p.i. (c,d) Percentages of cells 7 d.p.i. positive for IL-10–enhanced green fluorescent protein (eGFP) in influenza-specific PA224 or NP366 tetramer⁺ cells from Vert-X mice infected with H2-AbI−/− after 2 d in culture. Neutralization of mouse IL-2 completely blocked the induction of IL-10-producing CL-4 CTLs in vitro but only modestly affected cell proliferation and IFN-γ production by CTLs (Fig. 3a). Notably, addition of human IL-2 to cultures reversed the effects of mouse IL-2 neutralization (Fig. 3a). In companion experiments, we purified CD8⁺ T cells from double transgenic

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**Fig. 2** CD4⁺ T cell help is selectively required for the induction of IL-10-producing CTLs in vivo. (a,b) Production of IL-10, IFN-γ and TNF (a) by CTLs from MLNs or lungs of WT mice injected with rat immunoglobulin G (IgG) or anti-CD4 to deplete CD4⁺ T cells and infected with influenza, measured 8 d.p.i. by ICS. Numbers are the percentages of cells in gated population. (b) Normalized percentages of IL-10⁺ or TNF⁺ cells in influenza-specific CTLs (IFN-γ⁺) from lungs of WT and H2-AbI−/− mice infected with influenza, 7 d.p.i. (c,d) Normalized percentages of IL-10⁺ or TNF⁺ cells in influenza-specific CTLs (IFN-γ⁺) from lungs of WT and H2-AbI−/− mice infected with influenza, 7 d.p.i. (c,d) Percentages of cells 7 d.p.i. positive for IL-10–enhanced green fluorescent protein (eGFP) in influenza-specific PA224 or NP366 tetramer⁺ cells from Vert-X mice infected with H2-AbI−/− after 2 d in culture. Neutralization of mouse IL-2 completely blocked the induction of IL-10-producing CL-4 CTLs in vitro but only modestly affected cell proliferation and IFN-γ production by CTLs (Fig. 3a). Notably, addition of human IL-2 to cultures reversed the effects of mouse IL-2 neutralization (Fig. 3a). In companion experiments, we purified CD8⁺ T cells from double transgenic

**Fig. 3** IL-2 provides the help from CD4⁺ T cell to CTLs for IL-10 production in vitro. (a) Production of IL-10 and IFN-γ by CL-4 cells, measured through ICS. CFSE-labeled CD8⁺ CL-4 cells were stimulated with influenza-infected DCs in the absence or presence of hemagglutinin–specific CD4⁺ TCR transgenic T cells (TS-1). CL-4 T cells stimulated with influenza-infected DCs expanded and expressed IFN-γ but failed to produce IL-10 (Fig. 3a). However, when CD4⁺ TS-1 cells were also included in the culture, CL-4 cells acquired the ability to produce IL-10. Notably, all IL-10-producing cells were also IFN-γ⁺ (Supplementary Fig. 9). These data recapitulated our in vivo findings that CD4⁺ T cells were able to provide the help to CD8⁺ T cells to induce the development of IL-10-producing CTLs.

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ovalbumin (OVA)-specific TCR OT-I and Vert-X mice (V-OT-I) mice (V-OT-I) and cultured the CD8+ T cells with DCs simultaneously reconstituted with recombinant influenza strains expressing MHC type I or MHC type II OVA epitopes (influenza-OVA) in the presence or absence of OVA-specific CD4+ TCR transgenic T cells (OT-II) T cells. The inclusion of OT-II cells enhanced the development of IL-10-producing V-OT-I CTLs, which was dependent on IL-2 but independent of IL-6, IL-12 or IL-21 (Fig. 3b and Supplementary Fig. 10a). We further established that CD4+ T cells were the principal IL-2-producing cells in the culture (Fig. 3c). IL-2 is therefore likely to be the primary molecule derived from CD4+ T cells that provides help to CD8+ T cells to drive the development of IL-10-producing CTLs.

Our finding (Supplementary Fig. 4) that depletion of CD4+ T cells as late as 6 d.p.i. inhibited IL-10 production by CTLs in the infected lungs suggested that CD4+ T-cell–derived IL-2 may not only act at the induction phase of the CD8+ T cell response in the draining MLNs but also on previously activated CTLs in the lungs. To further explore this, we purified V-OT-I and OT-II cells and activated the transgenic CD8+ and CD4+ T cells independently in culture for 4 d, and then assessed the effect of IL-2 supplementation and/or activated CD4+ T cell addition on IL-10 production by the V-OT-I CTLs (Supplementary Fig. 10b). Antibody-mediated TCR activation of V-OT-I alone modestly upregulated IL-2-dependent expression of IL-10 (Fig. 3d, top row). IL-10 expression was further enhanced by the addition of exogenous IL-2 (Fig. 3d, top row). By contrast, culture with previously activated CD8+ and CD4+ T cells resulted in robust IL-10 expression by CTLs upon TCR cross-linking (Fig. 3d, bottom row) that was inhibited by IL-2 neutralization (Fig. 3d, bottom row). This finding supports the view that activated CD4+ T cells acting by means of IL-2 can promote the production of IL-10 by differentiated effector CD8+ T cells (CTLs). In keeping with this concept, only effector CD4+ T cells showed robust production of IL-2 (Fig. 3e). When these in vitro–activated CD8+ CTLs were treated with IL-2 alone in short-term culture (4 h), IL-2 exposure, like TCR engagement, was able to stimulate IL-10 expression by CTLs, but the IL-2 effect was more potent when signaling in cooperation with TCR engagement (Fig. 3f), suggesting that IL-2 can directly promote the expression of IL-10 independent of its role in promoting CTL proliferation and survival.

IL-2 signaling is required to induce IL-10+ CTLs in vivo

We next assessed the contribution of CD4+ T cell–derived IL-2 and IL-2 signaling to the induction of IL-10 production by antiviral CTLs in vivo during influenza infection. We first established in vivo that CD4+ T cells were the dominant source of IL-2 (Fig. 4a,b and Supplementary Fig. 11a) and that the depletion of CD4+ T cells abrogated most IL-2 production in both MLNs and the lung (Fig. 4c). Furthermore, the absence of CD4+ T cells impaired IL-10 but not IL-2 production by CD8+ T cells (Supplementary Fig. 11b), suggesting that the amount of IL-2 produced by CD8+ T cells was not sufficient to drive the maximal production of IL-10 by CTLs in vivo. In addition, transfer of wild-type (WT) CD4+ T cells but not IL2−/− CD4+ T cells with WT CD8+ T cells into Rag2−/− mice resulted in augmented IL-10 production by CTLs (albeit at lower levels than observed with CTLs from infected WT mice, likely owing to nonspecific homeostatic proliferation of transferred CD8+ T cells in Rag2−/− recipients), suggesting that CD4+ T cell–derived IL-2 is required for the optimal generation of IL-10-producing CTLs in vivo ( Supplementary Fig. 12).

In addition, we determined that IL-10-expressing CTLs in the infected lungs were IL-2Rα+ (CD25+), indicating that they are competent for IL-2 signaling (Supplementary Fig. 13).

To directly establish the requirement for IL-2 receptor signaling, we transferred either WT or IL2ra−/− T cells into congenic mice mismatched for the glycoprotein Thy-1 and then infected the recipient
mice with influenza. Whereas transferred WT CD8\(^{+}\) T cells or endogenous WT CD8\(^{+}\) T cells produced IL-10 in the infected lungs, transferred Il2ra\(^{-}\) CD8\(^{+}\) T cells failed to produce IL-10 during influenza infection (Fig. 4d). We also made WT:Il2ra\(^{-}\) mixed bone marrow chimeric mice and infected these mice with influenza. Il2ra\(^{-}\)-CTLs mounted an antigen-specific IFN-γ response that was moderately diminished compared to that in WT cells. In contrast, Il2ra\(^{-}\)-CTLs failed to produce IL-10 even when we accounted for the diminished proinflammatory cytokine response by normalizing the number of IL-10-producing cells to the number of antigen-specific IFN-γ-producing cells (Fig. 4e,f). By contrast, the Il2ra\(^{-}\) CTLs were able to produce equivalent amounts of TNF and only slightly diminished amounts of granzyme B compared to WT CTLs in the lung (Fig. 4f and Supplementary Fig. 14). IL-21 is another important CD4\(^{+}\) T cell–derived ‘helper’ cytokine. However Il21r\(^{-}\) CTLs were able to produce IL-10 at levels comparable to those of WT CTLs when influenza infection was carried out in the WT:Il21r\(^{-}\) mixed bone marrow chimeric mice (Fig. 4g). These data collectively demonstrate that IL-2 (rather than IL-21) is the main CD4\(^{+}\) T cell–derived molecule driving the development of IL-10-producing CTLs during influenza infection. Furthermore, rIL-2 administration to influenza-infected CD4\(^{+}\) T cell–depleted mice at the time of effector T cell infiltration into the lung—that is, 5–6 d.p.i.—enhanced the induction of IL-10 production by CTLs (Fig. 4h,i). Taken together, these data strongly suggest that IL-2 and IL-2 receptor–dependent signaling is critical for stimulating IL-10 production by CTLs at the site of virus infection.

**IL-2 and IL-27 cooperate to induce IL-10\(^{+}\) CTLs**

Our analysis so far indicated that both CD4\(^{+}\) T cell–derived IL-2 and the cytokine IL-27 were necessary for the development of IL-10-producing CTLs during influenza infection. Next, we sought to determine whether IL-27 and CD4\(^{+}\) T cell–derived IL-2 act synergistically to activate IL-10 gene expression by CTLs. To this end, we again cultured CFSE-labeled CL-4 cells with influenza-infected DCs in the presence or absence of TS-1 cells. Inclusion of TS-1 cells or IL-27 alone in the culture enhanced the induction of IL-10-producing CTLs (Fig. 5a). However, the presence either of TS-1 cells plus IL-27 or IL-2 plus IL-27 together in culture with the CD8\(^{+}\) T cells had a synergistic effect on the induction of IL-10-producing CTLs (Fig. 5a). Likewise, the synergy between IL-27 and CD4\(^{+}\) T cells depended primarily on the CD4\(^{+}\) T cell–derived IL-2 (Fig. 5a). Although CD4\(^{+}\) T cells are the main source of IL-2, CD8\(^{+}\) T cells are capable of producing a small amount of IL-2 during activation (Fig. 3c). This small amount of IL-2 produced by CD8\(^{+}\) T cells alone was insufficient to drive the development of IL-10-producing CTLs. However, in the absence of CD4\(^{+}\) T cell–derived IL-2, the small amount of CTL-derived IL-2 was essential for enhancing the effect of IL-27 on induction of IL-10-producing CTLs (Fig. 5a), suggesting that the induction of IL-10-producing CTLs by IL-27 likewise requires the presence of IL-2. The synergy between IL-27 and CD4\(^{+}\) T cell–derived IL-2 was also evident at the single cell level (Fig. 5b). Furthermore, IL-27 and IL-2 acted synergistically to induce IL-10-producing CTLs in the OT-I & OT-II culture system during the initial (primary) activation (Supplementary Fig. 15) or at the effector stage (Supplementary Fig. 16).

Having established the contribution of CD4\(^{+}\) T cells acting through IL-2 to stimulate IL-10 expression by CD8\(^{+}\) T cells, we wanted to probe the source(s) of the IL-27 in vivo. Innate immune cells in the infected lungs were the main source of IL-27 (Fig. 5c). In this regard, it is noteworthy that neutrophils may be a principal source of IL-27. Consistent with the finding that production of IL-10 by CTLs was largely restricted to the site of infection, potential IL-27-producing cells including neutrophils and monocytes or macrophages, as well as Il27 mRNA, were enriched at the infection site compared to their abundance in the draining MLNs (Supplementary Fig. 17). Furthermore, CD4\(^{+}\) cell depletion did not affect IL-27 production in vivo (Fig. 5d). This confirms that the impaired induction of
IL-10-producing CTLs in CD4+ T cell–depleted mice was not due to the lack of IL-27. IL-27 deficiency also did not impair IL-2 production by T cells (Supplementary Fig. 18), suggesting that the impaired induction of IL-10-producing CTLs in IL-27–deficient mice was not due to a lack of IL-2 production. Taken together, these data demonstrate that innate cell–derived IL-27 and CD4+ T cell–derived IL-2 were independently regulated but acted in a coordinate manner to induce IL-10 production by CTLs in the respiratory tract during influenza infection.

The unexpected finding of cooperation between these two cytokines in the mouse system prompted us to investigate whether IL-2 and IL-27 likewise act cooperatively to induce IL-10 production by human CD8+ T cells. IL-2 and IL-27 cooperatively induced both Il10 mRNA expression and IL-10 protein production by CD8+ T cells (Supplementary Fig. 19 and Fig. 5c).

Induction of IL-10-producing CTLs requires Blimp-1

To begin to define the signaling pathways within CTLs controlling the induction of IL-10 gene expression, we investigated transcription factors potentially implicated in controlling IL-10 production by CTLs. IL-10–expressing cells had higher mRNA levels of the transcriptional regulator Blimp-1 (the product of the gene Prdm1) than IL-10–negative CD8+ T cells (Fig. 6a). Blimp-1 expression is known to be induced by IL-2, and Blimp-1–deficient CD4+ T cells show comparable production of IFN-γ and IL-4 but diminished IL-10 in response to TCR stimulation.25,26 We therefore investigated the role of Blimp-1 in the production of IL-10 by CTLs. CD8+ T cells from infected lungs of H2-Ab1+/− mice expressed significantly less Prdm1 and Il10 (but not Il12 or Tbx21, encoding the transcription factor T-bet) than T cells isolated from littermate controls (Fig. 6b). Similarly, T cells from Ebi3−/− mice expressed less Prdm1 and Il10 than controls, but comparable Il12 and Maf (Fig. 6c). Furthermore, although exposure to either IL-2 or IL-27 was able to enhance Prdm1 expression in CTLs, simultaneous treatment of CTLs with both cytokines acted synergistically to further enhance this expression (Fig. 6d).

We then ectopically expressed Blimp-1 in CD8+ T cells by transducing CD8+ T cells with a Blimp-1–expressing retrovirus. Ectopic expression of Blimp-1 enhanced IL-10 production by CTLs (Fig. 6e), although it did not induce IL-10 production by CTLs as effectively as IL-2 plus IL-27 (data not shown), suggesting that other factors may contribute to maximal IL-10 production by CTLs. We next wished to determine whether IL-2– or IL-27–induced IL-10 production by CTLs requires Blimp-1 expression. CTLs. To this end, we isolated CD8+ T cells from WT mice or mice withloxP-flanked Prdm1 selectively disrupted in T cells (Cd4-Cre Prdm1fl/fl (Prdm1 cKO)) and stimulated them with DCs plus soluble antibody to CD3 (anti-CD3) in the presence or absence of human IL-2 or human IL-2 plus IL-27 for 4 d. (e) IL-10 production by OT-I cells, measured by ICS. OT-I cells were transduced with control human CD2 (hCD2)-expressing (vector) or hCD2–Blimp-1–expressing (Blimp-1) retrovirus, then cultured for 3 d. hCD2−, untransduced cells; hCD2+, transduced cells. (f) IL-10 production by CTLs, measured by ICS. CFSE-labeled CD8+ T cells from WT or Cd4-cre Prdm1fl/fl (Prdm1 cKO) mice were stimulated with DCs plus soluble anti-CD3 for 4 d under the conditions indicated at top. Numbers are the percentages of cells in the gated population. Data are representative of at least two independent experiments. *P < 0.05. Error bars, s.e.m.

The in vivo induction of IL-10+ CTLs requires Blimp-1

We next evaluated whether Blimp-1 is required for IL-10 production by CTLs in vivo. To do this, we infected Prdm1 cKO mice with influenza and at 7 d.p.i. measured IL-10 production by CTLs in the MLNs and infected lungs. Compared to CD8+ T cells from influenza-infected Blimp-1–sufficient (Cd4-Cre Prdm1fl/fl, control) mice, Blimp-1–deficient CD8+ T cells showed markedly diminished IL-10 production (Fig. 7a,b) with comparable production of IFN-γ and TNF upon influenza infection (Fig. 7a,b). The selective deficit in IL-10 production was also evident in bronchoalveolar lavage fluid (BALF) of the infected Prdm1 cKO mice (Fig. 7c). The diminished release of IL-10 into the BALF probably also reflects defective IL-10 production by CD4+ T cells, as the production of IL-10 by CD4+ T cells was also impaired in Prdm1 cKO mice (data not shown). Prdm1 cKO mice also showed enhanced pulmonary inflammation in response to infection, characterized by increased infiltration of the infected lungs by mononuclear and granulocytic inflammatory cells and enhanced production of proinflammatory cytokines similar to that seen upon IL-10R blockade6 (Fig. 7d and Supplementary Fig. 20).
DISCUSSION

CD4+ T-cell help for CD8+ T-cell responses has been previously demonstrated to be required for memory CTL formation, maintenance and recall responses27–29, in CTL migration to mucosal surfaces30 and in certain experimental settings for optimum primary CTL responses to weak stimuli31–33. Strong inflammatory stimuli such as influenza infection, however, trigger potent primary CTL responses independent of CD4+ T-cell help19,20. Our results support this picture, as CD4+ T-cell help was minimally required for expression of type I effector cytokines such as IFN-γ and cytolytic machinery such as granzyme B by CTLs in vivo. However, without CD4+ T-cell help, antiviral CTL activation and differentiation is incomplete because CTLs are unable to optimally produce IL-10, a critical regulatory cytokine necessary to control excess lung inflammation during immune-mediated virus clearance6.

IL-2 has been shown to promote CTL memory and to support terminal effector cell differentiation by upregulating cytolytic molecules; for example, granzyme B in secondary lymphoid organs23,34,35. Our findings on the effect of defective IL-2 signaling on granzyme B expression by proliferating and/or differentiating CTLs in the draining MLNs support these earlier findings23,34,35. However, within the inflammatory milieu of the infected lungs, other signals can replace IL-2 in the induction of CTL granzyme B. Proinflammatory molecules such as type I interferons, which can induce antigen-independent granzyme B expression in CTLs within the respiratory tract26, may replace IL-2. By contrast we find that IL-2 has a critical nonredundant role in promoting the induction of IL-10-producing CTLs.

Here we also demonstrate a synergistic role for IL-27 in the CD4+ T-cell–mediated induction of IL-10 by CTLs. Although recognized as a stimulator of type 1 helper T cell responses, IL-27 has more recently been appreciated as an important IL-10-independent and IL-10–dependent regulator of host immune responses27—for example, CD4+ Tr1 and CD4+ effector cells induce IL-10 expression in vivo10,14,15. Our findings suggest that antiviral CTLs within the virally infected lungs are also targets of IL-27 in vivo. Although IL-27 is generally believed to be the product of activated macrophages and DCs38, our results suggest that neutrophils may also be an important source of IL-27 during acute respiratory viral infections. This interplay between inflammatory myeloid cell–derived IL-27 and CTL–derived IL-10 suggests a new type of regulatory loop whereby influx of inflammatory myeloid cells into the infected lungs in response to infection augments IL-10 production by CTLs responding to infection, which in turn dampens both the activation state of the innate immune cells and further infiltration of these myeloid inflammatory cells2.

Blimp-1 is a transcriptional repressor that functions in B cell differentiation, as well as T cell homeostasis, T effector cell differentiation and migration39. As previously reported40, Blimp-1–deficient CTLs showed a modest deficit in migration into the influenza–infected lungs, but the Blimp-1–deficient CTLs within the infected lungs showed no deficit in proinflammatory effector cytokine production. Blimp-1–deficient CTLs were, however, markedly impaired in IL-10 production, suggesting stringent requirements for Blimp-1 expression by IL-10–producing CTLs in vivo in the infected lung. Consistent with this hypothesis, IL-2– and/or IL-27–dependent IL-10 production by CTLs in vitro required that these cells express Blimp-1. Of note, both IL-10 nonproducing (IL-10−) and IL-10+ CTLs expressed Blimp-1, but the IL-10− CTLs expressed two to three times as much Blimp-1. Thus a modest difference in the expression of this transcriptional regulator seems to markedly influence the ability of the activated CTLs to express the IL-10 gene, a result consistent with recently published
evidence relating Blimp-1 gene dosage to CD8+ T cell exhaustion. Although our findings indicate a likely nonredundant requirement for both Blimp-1 and IL-2 to generate IL-10-producing CTLs, both IL-10+ and IL-10− CTLs express Blimp-1 and are exposed to IL-2 and IL-27 in the infected lungs. Undoubtedly other, as yet unidentified factors also affect IL-10 gene expression by CTLs in the infected respiratory tract.

In summary, we have described the cellular and molecular mechanisms controlling a regulatory cytokine production by antiviral CTLs. Our data reveal coordination between innate cell-derived signal and helper T cell-derived signal in fine-tuning the antiviral CTLs responses during acute respiratory virus infection. Our findings may provide the groundwork for future studies in manipulating IL-10 production in antiviral T cells to control excessive host inflammation during acute respiratory virus infection.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank the rest of Braciale laboratory for critical comments and B. Small for excellent technical assistance. We thank C.L. Karp (Cincinnati Children’s Hospital Medical Center), M. Mohrs (Trudeau Institute), W. Leonard (US National Institutes of Health) and T.R. Malek (University of Miami) for reagents. This work was supported by the US National Institutes of Health (grants AI-15608, HL-33391, AI-37293 and U19 AI-083024 to T.J.B.), University of Virginia Center for Immunity, Inflammation and Regenerative Medicine start-up funds to R.S., and an American Lung Association Postdoctoral Fellowship (RN-123000) to J.S.

AUTHOR CONTRIBUTIONS

J.S. designed the project, performed most of the experimental work, analyzed the data and wrote the manuscript. H.D. and E.K.M. performed some of the quantitative RT-PCR and ELISA experiments. R.S. contributed to reagents and suggestions. T.J.B. supervised the project, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and infections. C57BL/6, BALB/c, Rag2<sup>−/−</sup>, Eβ<sup>−/−</sup>, Ifng<sup>−/−</sup>, Il12<sup>−/−</sup>, OT-II, Pbm1<sup>fl/fl</sup>, H-2<sup>B</sup>-Ab1<sup>−/−</sup> and Cd4-cre transgenic mice were purchased from Taconic Farm or Jackson Laboratories. Vert-X mice<sup>21</sup> were from C. Karp, Il21r<sup>−/−</sup> mice were from W. Leonard, and Yeti mice<sup>22</sup> were from M. Mohrs. CL-4, TS-1, Il2<sup>−/−</sup>, Il2<sup>−/−</sup>, H-2<sup>B</sup>-Ab1<sup>−/−</sup>, Vert-X, and Vert-X OT-I mice were bred in house. All mice were housed in a specific pathogen–free environment and all animal experiments were performed in accordance with protocols approved by the University of Virginia Animal Care and Use Committee. For virus infection, mice were infected with sublethal dose of influenza strain A/PR/8/34 in serum-free Iscove’s medium intranasally after anesthesia with ketamine and xylazine.

Quantitative RT-PCR. Lung cell suspensions were prepared as described<sup>42</sup>. CD8<sup>+</sup> cells were purified through magnetic-activated cell separation (MACS) beads (Miltenyi). mRNA isolation, reverse transcription and real-time PCR were performed as previously described<sup>6</sup>. Data were generated with the comparative threshold cycle method by normalizing to hypoxanthine phosphoribosyltransferase (HPRT).

Influenza-specific CD8<sup>+</sup> T cell restimulation by bone marrow DCs. Bone marrow DCs were collected on day 6–7 of culture with granulocyte–macrophage colony-stimulating factor and infected with influenza at a multiplicity of infection of ~100 overnight. Then DCs were counted and mixed with lung or MLN cells at a 1:5.1 ratio in the presence of Golgi-Stop (BD Biosciences) and human IL-2 (40 U ml<sup>−1</sup>) for another 6–7 h. The surface staining of cell surface markers and intracellular staining of cytokines were performed as described<sup>8</sup>.

DC–T cell culture. CD4<sup>+</sup> and CD8<sup>+</sup> transgenic T cells were isolated from spleen and lymph nodes of mice using MACS beads (Miltenyi). Splenic DCs were isolated from spleens of WT mice using MACS beads (Miltenyi). Then DCs were infected at multiplicity of infection of ~100. For CL-4 and TS-1 experiments, DCs were infected with influenza A/PR8; for OT-I/OT-II experiments, DCs were infected with recombinant influenza A/PR8-OT-I plus A/PR8-OT-II virus). DCs were then mixed with carboxylfluorescein diacetate succinimidyl ester (CFSE)-labeled CD8<sup>+</sup> T cells (5 × 10<sup>5</sup>) at the ratio of 1:DC10 T cells in round-bottom 96-well plates. In some wells, we included the same number of CD4<sup>+</sup> T cells. The conditions of the culture are indicated in the text. For experimental cultures with WT or Blimp-1-deficient CD8<sup>+</sup> T cell, we stimulated T cells with soluble anti-CD3 (145-2C11, BD Biosciences, 0.05 µg ml<sup>−1</sup>) with influenza-infected bone marrow DCs at the ratio of 1:DC10 T cells. The T cells were cultured for 4 d and restimulated with either cognate peptide (1 µg ml<sup>−1</sup>) or phorbol 12-myristate 13-acetate (100 ng ml<sup>−1</sup>) plus ionomycin (1 µg ml<sup>−1</sup>) in the presence of Golgi-Stop (1 µl ml<sup>−1</sup>). For secondary cultures of activated effector T cells, V-OT-I or OT-II cells were activated separately as described above. Then 5 × 10<sup>4</sup> V-OT-I cells or V-OT-I (5 × 10<sup>5</sup>) plus OT-II cells (5 × 10<sup>5</sup>) were left unstimulated or stimulated with plate-bound anti-CD3 (coated with 50 µl of 1 µg ml<sup>−1</sup> in PBS for 3–4 h in 37 °C) for 2 d. All blocking antibodies were used at 20 µg ml<sup>−1</sup>. Recombinant human IL-2 and mouse IL-27 were used at 300 U ml<sup>−1</sup> and 10 ng ml<sup>−1</sup>. Then the culture supernatant were collected for human IL-10 ELISA and cells were collected for quantitative RT-PCR analysis of Il10 expression.

Bone marrow chimeras. To generate WT and Il2ra<sup>−/−</sup> mixed bone marrow chimeras, we lethally irradiated (1,020 rad) WT mice and reconstituted the irradiated mice with Thy-1.1<sup>+</sup> WT bone marrow mixed with Thy-1.2<sup>+</sup> Il2ra<sup>−/−</sup> bone marrow. To generate WT and Il2<sup>−/−</sup> mixed bone marrow chimeras, we lethally irradiated (1,020 rad) WT mice and reconstituted the irradiated mice with CD45.1<sup>+</sup> WT bone marrow mixed with CD45.2<sup>+</sup> Il2<sup>−/−</sup> bone marrow. After 8–10 weeks, the reconstituted mice were then infected with influenza.

Cell transfer and infection. For T cell transfer into WT mice, cells were isolated from Thy-1.2<sup>+</sup> WT, Il2ra<sup>−/−</sup> or Cd4-cre Pbm1<sup>fl/fl</sup> spleens and lymph nodes. A total of 50 million cells were then transferred into Thy-1.1<sup>+</sup> mice. The recipient mice were infected with influenza 24 h later. For CD4<sup>+</sup> and CD8<sup>+</sup> T cell transfer into RAG2<sup>−/−</sup> mice, 15 million purified WT CD8<sup>+</sup> cells were either used alone or mixed with 30 million purified WT CD4<sup>+</sup> cells or CD4<sup>+</sup> CD8<sup>+</sup> cells and then transferred into RAG2<sup>−/−</sup> mice. Recipient mice were infected with influenza 24 h later. At 9 d.p.i., lung cells were collected and analyzed by FlowJo software (Treestar).

Cell sorting. For experiments to measure IL-27 expression, WT mice were infected with influenza and different cell populations were sorted based on the following markers at 5 d.p.i.: neutrophils, CD11b<sup>+</sup>Ly6G<sup>+</sup>; DCs, MHCII<sup>+</sup>CD11c<sup>+</sup>; monocytes and macrophages, CD11b<sup>+</sup>MHCII<sup>−</sup>CD11c<sup>−</sup> or low; T cells, Thy-1.2<sup>+</sup>; lung resident cells, CD45<sup>+</sup>. For effector T cells sorting from infected Vert-X mice, we sorted CD4<sup>+</sup>IL-10–eGFP<sup>+</sup> or CD4<sup>+</sup>IL-10–eGFP<sup>+</sup> CD8<sup>+</sup> T cells from lungs 7 d.p.i.

Retroviral transduction. Control human CD2 (hCD2)-containing pMI retroviral vector and hCD2–Blimp-1–containing pMI retroviral vector were a gift from T.R. Malek<sup>25</sup>. Retroviral supernatants were generated by transient transfection of HEK-293T cells with TransIT-LT1 reagent (Mirus) in the presence of pCL-Eco Retrovirus packaging plasmid. Retrovirus-containing supernatants were collected 48 h after transfection and used for spin infection (1,260 g, 2 h) of preactivated OT-I cells (bone marrow DCs plus 1 µg ml<sup>−1</sup> of OVA peptide for 24 h). Then cells were washed and placed into culture with influenza-OVA–infected DCs for another 3 d.

Antibodies and flow cytometry analysis. All antibodies were purchased from BD Biosciences or eBioscience (unless otherwise stated); CD4 (DK1.5, Bio-X-Cell, for in vivo depletion), CD4 (L3T4, for staining), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD25 (PC61), CD44 (IM7), CD45 (30-F11), CD90.1 (OX-7), CD90.2 (53-2.1), Ly6G (1A8), Ly6C (AL-21), IL-2 (18175A), IFN-γ (XMGI1.2), TNF (MP6-XT22) and IL-10 (JES5-16E3). Cells were sorted through a six-color FACS-Canto system (BD Biosciences). Data were then analyzed by FlowJo software (Treestar).

Statistical analysis. Data are mean ± s.e.m. A two-tailed Student’s t-test was used.


doi:10.1038/ni.1996