Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10

Jie Sun¹, Rajat Madan², Christopher L Karp² & Thomas J Braciale^{1,3}

Activated antigen-specific T cells produce a variety of effector molecules for clearing infection but also contribute to inflammation and tissue injury. Here we report an anti-inflammatory property of antiviral CD8⁺ and CD4⁺ effector T cells (T_{eff} cells) in the infected periphery during acute virus infection. We find that, during acute influenza infection, interleukin-10 (IL-10) is produced in the infected lungs in large amounts—exclusively by infiltrating virus-specific T_{eff} cells, with CD8⁺ T_{eff} cells contributing a larger fraction of the IL-10 produced. These T_{eff} cells in the periphery simultaneously produce IL-10 and proinflammatory cytokines and express lineage markers characteristic of conventional T helper type 1 or T cytotoxic type 1 cells. Notably, blocking the action of the T_{eff} cell–derived IL-10 results in enhanced pulmonary inflammation and lethal injury. Our results show that antiviral T_{eff} cells exert regulatory functions—that is, they fine-tune the extent of lung inflammation and injury associated with influenza infection by producing an anti-inflammatory cytokine. We discuss the potential implications of these findings for infection with highly pathogenic influenza viruses.

The innate and adaptive immune systems have pivotal roles in orchestrating the host response to infection and tissue injury. The host responses to such insults include the production of a variety of proinflammatory cytokines and chemokines. The induction of the proinflammatory response triggers the development of a counter-regulatory anti-inflammatory cytokine response to control inflammation and prevent excessive injury. In certain instances (for example, infection with the highly pathogenic avian H5N1 or 1918 pandemic influenza virus strains), this counter-regulation fails, as infection results in a massive inflammatory cell infiltration into the infected lungs and excessive proinflammatory cytokine production^{1–3}.

IL-10 has been recognized as a major anti-inflammatory cytokine serving as a negative regulator of the response of both innate and adaptive immune cells, particularly during persistent bacterial and parasitic infections, where it suppresses pathogen clearance, infection-associated immunopathology or both^{4,5}. More recently, IL-10 has been found to mediate virus persistence during chronic viral infections^{6–8}. Multiple cell types have been reported to produce IL-10 either constitutively or in response to inflammatory stimuli, most notably regulatory T cells and dendritic cells and, recently, CD4⁺ effector T cells during protozoa infections^{4,9–12}. IL-10 has not been reported to play any considerable part in acute virus infections.

Here we analyzed the production, cellular source and function of IL-10 during acute respiratory influenza virus infection. To our surprise, adaptive immune CD8⁺ and CD4⁺ T_{eff} cells were the primary source of IL-10 produced in the influenza-infected respiratory tract, with CD8⁺ T_{eff} cells contributing larger fraction of the total IL-10

produced. IL-10 and proinflammatory cytokines were simultaneously produced by these T_{eff} cells early in the adaptive response. Blockade of the action of IL-10 *in vivo* in sublethally infected mice resulted in enhanced pulmonary inflammation (elevated inflammatory cell infiltration and cytokine and chemokine production), lethal injury and accelerated death, with no effect on the tempo of virus clearance. Lethal injury was partially reversed by corticosteroid administration. Our findings suggest that T_{eff} cell–derived IL-10 may have a crucial role in regulating the magnitude of inflammation during acute virus infection.

RESULTS

Influenza-specific T cells produce IL-10 in infected lungs

We analyzed cytokine secretion by CD4⁺ and CD8⁺ T cells infiltrating the lungs of influenza A/PR/8/34 virus-infected mice. We unexpectedly found that a substantial fraction of both CD4⁺ and CD8⁺ T cells from the infected lungs secreted IL-10, interferon- γ (IFN- γ) or both (and minimal IL-4 or IL-17) in response to phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation in the intracellular cytokine synthesis (ICS) assay (**Supplementary Fig. 1** online). Furthermore, the mitogen-induced IL-10 synthesis was restricted to Thy-1⁺ lymphocytes in the lungs (**Supplementary Fig. 2** online).

We next compared the levels of IL-10 and IFN- γ transcripts in purified CD3⁺ cells and the residual CD3[–] cells isolated from influenza-infected lungs. We observed that, like IFN- γ message expression, the expression of IL-10 transcripts was primarily restricted to the CD3⁺ cell fraction (**Fig. 1a**). We also detected the expression of

¹Beirne B. Carter Center for Immunology Research, University of Virginia, 409 Lane Road, Charlottesville, Virginia 22908, USA. ²Division of Molecular Immunology, Cincinnati Children's Hospital Research Foundation, and the University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA.

³Departments of Microbiology and Pathology, University of Virginia, 409 Lane Road, Charlottesville, Virginia 22908, USA. Correspondence should be addressed to T.J.B. (tjb2r@virginia.edu).

Figure 1 Influenza-specific T cells preferentially produce IL-10 and IFN- γ in the influenza-infected BALB/c lungs. **(a)** Quantitative RT-PCR analysis of IL-10 and IFN- γ transcript levels in purified CD3⁺ and CD3⁻ cells in the lungs of mice 8 d after infection. ND, not determined. **(b,c)** Quantitative RT-PCR analysis of IL-4, IL-17, IL-10 and IFN- γ transcript levels of purified CD8⁺ **(b)** or CD4⁺ **(c)** T cells in the lungs of mice 8 d after infection. **(d)** ICS at day 8 after infection of lung, MLN and spleen cells re-stimulated *in vitro* with influenza-infected BMDCs to measure the production of IL-10 and IFN- γ in gated CD8⁺ and CD4⁺ T cells. Numbers indicate the percentages of cytokine-positive cells in the gates within the total population. **(e,f)** ICS to measure the production of IL-10 and IFN- γ in gated CD8⁺ **(e)** and CD4⁺ **(f)** T cells from MLN and lungs of mice 8 d after infection that were re-stimulated with flu-infected BMDCs. The ratio of IL-10⁺ versus IFN- γ ⁺ CD8⁺ **(e)** or CD4⁺ **(f)** T cells and the mean fluorescence intensity (MFI) of IL-10 staining in IL-10⁺CD8⁺ **(e)** or IL-10⁺CD4⁺ **(f)** T cells in MLN and lungs are depicted. *P* values were determined by unpaired two-tailed Student's *t* test. Values are means $\pm$ s.d.

IL-10 messenger RNA in both purified CD8⁺ (**Fig. 1b**) and CD4⁺ (**Fig. 1c**) T cells isolated from the infected lungs, whereas minimal levels of IL-4 and IL-17 mRNA were detected in either T cell subset (**Fig. 1c**). To determine whether lung T cells are capable of producing IL-10 in response to viral antigen, we used the ICS assay to examine IL-10 and IFN- γ production by CD8⁺ and CD4⁺ T cells isolated from lungs, the draining mediastinal lymph nodes (MLN) and spleens of mice 8 d after infection by stimulation with influenza-infected bone marrow-derived dendritic cells (BMDCs). We found that a substantial fraction of CD8⁺ and CD4⁺ T cells from the infected lungs and a smaller fraction of CD8⁺ and CD4⁺ T cells from MLN and spleens were capable of producing IL-10 in response to viral antigen (**Fig. 1d**), with a higher percentage of antigen-specific CD8⁺ and CD4⁺ T cells (as indicated by the IFN- γ production) in the lungs capable of producing IL-10 than among those detected in the MLN (**Fig. 1e,f**). Furthermore, the IL-10-producing T cells in the lungs expressed higher amounts of IL-10 than the corresponding cells in the MLN (**Fig. 1e,f**). We also detected the induction of IL-10-producing CD8⁺ T cells with a recall response to heterologous influenza virus challenge and during primary respiratory syncytial virus infection (**Supplementary Fig. 3** online and data not shown).

IL-10-producing T cells are CD8⁺ and CD4⁺ T_{eff} cells

IL-10 production has typically been detected in CD4⁺ effector and regulatory T cells⁹. Our observation that a substantial fraction of antigen-specific CD8⁺ T cells at the site of infection in the lung were capable of responding to antigen with IL-10 production was unexpected and, to our knowledge, has not been previously shown. To determine the phenotype of these IL-10 producing (IL-10⁺) CD8⁺ T cells, we first examined the expression of the type 1 T_{eff} lineage-specific transcription factor T-bet and the regulatory T cell lineage-specific transcription factor Foxp-3 by the IL-10⁺CD8⁺ T cells, the 'conventional' CD8⁺ IFN- γ ⁺ cytotoxic type 1 (T_{C1}) T_{eff} cells, and CD8⁺IL-10⁻IFN- γ ⁻ T cells. Like T_{C1} T_{eff} cells, the IL-10⁺ T cells expressed high amounts of T-bet but not the regulatory cell marker

Foxp-3 (**Fig. 2a,b**). Further confirmation that these IL-10-producing T cells were T_{C1} T_{eff} cells came from the discovery that these IL-10⁺ T cells express effector molecules (for example, granzyme B, CD107a and tumor necrosis factor- α (TNF- α)) characteristic of conventional T_{C1} T_{eff} cells (**Fig. 2c**). Moreover, IL-10-producing CD8⁺ T cells were also capable of proliferating on-site in the infected lungs, as measured by a 2-h BrdU incorporation assay (**Fig. 2d**), suggesting that, in contrast to regulatory IL-10-producing CD4⁺ T cells¹³, they were not anergic *in vivo*. We also established that the majority of the IL-10-producing CD4⁺ T cells had a phenotypic profile characteristic of effector T helper type 1 T_{eff} cells (**Fig. 2e,f**), although a very small fraction of the IL-10-producing CD4⁺ T cells also expressed T-bet and Foxp-3 and thus showed the phenotype of the regulatory T cells described previously¹⁴ (**Fig. 2e,f**). Like the IL-10-producing CD8⁺ T_{eff} cells, IL-10-producing CD4⁺ T cells were capable of proliferating *in situ* in the infected lungs (**Fig. 2g**).

CD4⁺ but not CD8⁺ memory T cells express IL-10

We next examined the kinetics of IL-10 expression by virus-specific CD8⁺ and CD4⁺ T cells in infected lungs. Consistent with the timing of CD8⁺ T_{eff} cell migration during influenza infection, IL-10⁺ lung CD8⁺ T_{eff} cells were first readily found at day 6 after infection (**Fig. 3a**). The total number of IL-10⁺ and IFN- γ ⁺ CD8⁺ T_{eff} cells in the lungs continued to increase until day 10 after infection, and CD8⁺ T_{eff} cells capable of secreting IL-10 were still detectable during the early contraction phase of the response (that is, day 15; **Fig. 3b**). However, with additional contraction of the response and the transition into the memory phase (that is, on day 26 and beyond), the fraction of IL-10-producing T cells in the remaining virus-specific (IFN- γ ⁺) CD8⁺ T cells was markedly decreased and eventually became undetectable (**Fig. 3a,b**).

The kinetics of accumulation of IL-10-expressing CD4⁺ T_{eff} cells into infected lungs directly paralleled that of IL-10-producing CD8⁺ T_{eff} cells (**Fig. 3c,d**). IL-10 production was largely restricted to IFN- γ ⁺CD4⁺ T_{eff} cells, but, in contrast to CD8⁺ T_{eff} cells, a fraction of

Figure 2 IL-10-producing CD8⁺ and CD4⁺ T cells are type 1 effectors. BALB/c mice were infected with influenza and lung cells were re-stimulated with influenza-infected BMDCs. (**a–c**) The expression of T-bet (**a**), Foxp-3 (**b**) and a panel of activated T cell markers (Gzmb, CD107a, TNF- α , IL-2, CTLA4, 1B11, CD44, CD11a, PD-1 and CD27) (**c**) in uninfected lung CD8⁺ T cells (control) or IL-10-positive (IL-10⁺), IFN- γ single-positive (IFN- γ SP) or IL-10 and IFN- γ double-negative (DN) CD8⁺ T cells in the lung 7 d after infection, as measured by ICS. (**d**) Uninfected mice (control) and mice 6 d after infection were injected with BrdU. The percentages of BrdU⁺ cells in control CD8⁺ T cells or the IL-10⁺, IFN- γ SP or DN CD8⁺ T cells of the lung 6 d after infection are depicted. (**e,f**) The expression of T-bet (**e**) and Foxp-3 (**f**) in control CD4⁺ T cells or IL-10⁺, IFN- γ SP or DN CD4⁺ T cells of the lung 7 d after infection, as measured by ICS. (**g**) Uninfected mice (control) and mice 6 d after infection were injected with BrdU. The percentages of BrdU⁺ cells in control CD4⁺ T cells or the IL-10⁺, IFN- γ SP and DN CD4⁺ T cells of the lung 6 d after infection are depicted. For **a** and **e** the gray line indicates isotype control antibody staining. Numbers are MFI (**a,e**) or percentages of Foxp-3⁺ cells in the gates within the total population (**b,f**). Values are means $\pm$ s.d.

CD4⁺ T_{eff} cells were IL-10 single positive (Fig. 3c). In a further contrast with CD8⁺ T_{eff} cells, virus-specific IL-10⁺CD4⁺ memory T cells were readily detected at day 26 after infection and remained detectable as late as day 95 (Fig. 3c,d).

T_{eff} cells produce IL-10 *in vivo* during influenza infection

To determine whether IL-10 was produced *in vivo*, we used ELISA to measure the kinetics of IL-10 and IFN- γ release into bronchial alveolar lavage fluid (BALF) sampled from infected lungs. We found that minimal amounts of either cytokine were detected in the BALF early during infection (day 4 after infection; Fig. 4a), in spite of high lung virus titers at this time¹⁵. By day 6 after infection, both IL-10 and IFN- γ concentrations in the BALF increased substantially and in a coordinated manner, with BALF concentrations of these two cytokines decreasing progressively through days 8 to 10 after infection and returning to background levels by day 16 (Fig. 4a). It is particularly noteworthy that this marked

rise in the concentrations of these two cytokines at day 6 coincided with the recruitment of CD8⁺ and CD4⁺ T_{eff} cells into the infected lungs¹⁶. Furthermore, the subsequent decline in the production of these two cytokines coincided with the fall in lung virus titers (and therefore viral antigen load)¹⁷, even though the absolute number of CD8⁺ and CD4⁺ T_{eff} cells continued to increase in the lungs up to day 10 after infection (Fig. 3b,d).

In support of the hypothesis that infiltrating T_{eff} cells are the main IL-10 producer *in vivo*, we found that minimal amounts of IL-10 and IFN- γ were produced in the BALF sampled from influenza-infected genetically T lymphocyte-deficient *Rag1*^{-/-} mice (Fig. 4b). Further confirmation of this hypothesis came from the analysis of IL-10 and

Figure 3 Kinetics of IL-10-producing CD8⁺ and CD4⁺ T_{eff} cells *in vivo*. BALB/c mice were infected with influenza. (**a–d**) At the indicated days after infection, lung cells were re-stimulated with influenza-infected BMDCs. The production of IL-10 and IFN- γ by CD8⁺ and CD4⁺ T cells was measured by ICS. (**a,c**) FACS plots of IL-10 and IFN- γ production by CD8⁺ (**a**) or CD4⁺ (**c**) T cells in the lung. Numbers indicate the percentages of cytokine-positive cells in the gates within the CD8⁺ (**a**) or CD4⁺ (**c**) T cell population. (**b,d**) The kinetics of accumulation (absolute numbers) of IL-10⁺ or IFN- γ ⁺ CD8⁺ T cells (**b**) or IL-10⁺ or IFN- γ ⁺ CD4⁺ T cells (**d**) in the lungs after influenza infection. Values are means $\pm$ s.d.

Figure 4 T_{eff} cells are the major source of IL-10 *in vivo* during influenza infection. BALB/c mice were infected with influenza (a–e). (a) IL-10 and IFN-γ concentrations in the BALF at the indicated days after infection, as measured by ELISA. (b) Wild-type (WT) and *Rag1*^{−/−} mice were infected with influenza. IL-10 and IFN-γ concentrations in the BALF at day 6 after infection, as measured by ELISA. Rag control, uninfected *Rag1*^{−/−} mice; Rag Flu, *Rag1*^{−/−} mice infected with influenza; WT Flu, WT mice infected with influenza. (c) IL-10 and IFN-γ concentrations in the BALF at day 6 after infection, as measured by ELISA. Control, uninfected mice; PBS, mice injected with PBS; anti-CD8, mice injected with CD8-specific mAb; anti-CD4, mice injected with CD4-specific mAb. (d) ICS was performed to measure the production of IL-10 and IFN-γ in gated CD8⁺ T cells from lung and MLN cells from control or CD4⁺ T cell-depleted mice at day 6 after infection and re-stimulated *in vitro* with influenza-infected BMDCs. Numbers indicate the percentages of cytokine-positive cells in the gates within the total population. (e) *In vivo* ICS assay to measure the production of IL-10 and IFN-γ by CD8⁺ and CD4⁺ T cells in the lung *in vivo* at day 6 after infection in mice injected with monensin. The numbers of IL-10⁺ CD8⁺, IL-10⁺ CD4⁺, IFN-γ⁺ CD8⁺ and IFN-γ⁺ CD4⁺ T cells are shown. *P* values were determined by unpaired two-tailed Student's *t* test. (f) IL-10-EGFP reporter mice were infected with influenza. At day 6 after infection, the numbers of IL-10-EGFP⁺ CD8⁺ and IL-10-EGFP⁺ CD4⁺ T cells in the lungs, MLN and spleens were measured by flow cytometry. Values are means ± s.d.

IFN-γ expression in the mice after selective depletion of CD8⁺ T cells, CD4⁺ T cells or both. The depletion of CD8⁺ T cells substantially decreased IL-10 and IFN-γ release into the BALF, suggesting that CD8⁺ T_{eff} cells are a key source of the two cytokines *in vivo* (Fig. 4c). The depletion of CD4⁺ T cells only slightly influenced the production of IFN-γ but markedly inhibited the production of IL-10 in BALF, suggesting that IL-10 production *in vivo* is also dependent on CD4⁺ T cells (Fig. 4c). IL-10 and IFN-γ release into the BALF was decreased to control levels by the simultaneous depletion of CD8⁺ and CD4⁺ T cells (Fig. 4c).

We were intrigued by the findings that lung IL-10 production is dependent on both CD8⁺ and CD4⁺ T cells during influenza infection *in vivo*. To investigate this phenomenon, we measured the induction of IL-10-producing CD8⁺ T_{eff} cells *in vivo* after the depletion of CD4⁺ T cells. We found that the CD4⁺ T cell depletion impaired the development of IL-10-producing CD8⁺ T_{eff} cells, but not IFN-γ- or TNF-α-producing T_{eff} cells (Fig. 4d and data not shown). Notably, the induction of IL-10-producing CD4⁺ T_{eff} cells was independent of CD8⁺ T cells (Supplementary Fig. 4 online). To directly delineate the contribution of CD4⁺ and CD8⁺ T_{eff} cells to IL-10 production *in vivo*, we performed the *in vivo* ICS assay and found that the numbers of IL-10-producing CD8⁺ T_{eff} cells were two- to threefold higher than those of IL-10-producing CD4⁺ T_{eff} cells in the lung (Fig. 4e and Supplementary Fig. 5 online), suggesting that CD8⁺ T_{eff} cells are a larger contributor to the T_{eff} cell-derived IL-10 in the infected lungs than CD4⁺ T_{eff} cells. We also monitored the induction of IL-10-producing cells in the lung after influenza infection of IL-10-EGFP reporter-expressing mice. In these mice, IL-10-EGFP⁺ cells in infected lungs were tightly restricted to the Thy1⁺ T lymphocyte population (Supplementary Fig. 6 online). Furthermore, although CD4⁺IL-10-EGFP⁺ cells predominated in the MLN and spleen, the number of lung CD8⁺IL-10-EGFP⁺ cells was more than twofold higher than the

number of lung CD4⁺IL-10-EGFP⁺ cells (Fig. 4f and Supplementary Fig. 7 online). Taken together, these data demonstrate that the production of IL-10 in influenza-infected lungs is exclusively restricted to the infiltrating T_{eff} cells, with CD8⁺ T_{eff} cells predominating over CD4⁺ T_{eff} cells as contributors to the T_{eff} cell-derived IL-10.

IL-10R blockade *in vivo* leads to lethal pulmonary injury

To test the function of IL-10 during influenza infection, we blocked IL-10 signaling *in vivo* by administration of a blocking IL-10 receptor (IL-10R)-specific monoclonal antibody (mAb) to mice undergoing sublethal infection with influenza. We found that blockade of IL-10 signaling resulted in increased and accelerated mortality (Fig. 5a). Of

Figure 5 IL-10R blockade *in vivo* results in increased mortality and accelerated death during influenza infection. (a) Survival of BALB/c mice infected with influenza and treated with PBS, rat IgG1 control mAb (rat IgG1) or IL-10R-specific mAb (anti-IL-10R). *P* value was determined by the log-rank survival test. (b) Airway BALF virus titers in BALB/c mice infected with influenza and treated with rat IgG1 control mAb or IL-10R-specific mAb at days 6 and 8 after infection. *P* value was determined by unpaired two-tailed Student's *t* test. NS, not significant. Values are means ± s.d.

Figure 6 IL-10R blockade leads to lethal pulmonary inflammation during influenza infection. **(a)** Fold changes of cytokines in the BALF of IL-10R mAb-treated mice versus those of cytokines in the BALF of Rat IgG1-treated mice infected with influenza. At day 8 after infection, cytokines in the BALF were determined by multiplex cytokine array analysis. p40 and p70 are IL-12 subunits MCP-1, monocyte chemoattractant protein-1; MIP-1 α , macrophage inflammatory protein-1 α . **(b,c)** Flow cytometry analysis of lung inflammatory monocytic cells **(b)** and neutrophils **(c)** in BALB/c mice infected with influenza and treated with IL-10R-specific mAb or rat IgG1 control mAb at days 4, 6 and 8 after infection. *P* value was determined by unpaired two-tailed Student's *t* test. **(d,e)** IL-12 p40 **(d)** and IFN- γ **(e)** concentrations in the BALF, as determined by ELISA, of BALB/c mice infected with influenza and treated with IL-10R-specific mAb or rat IgG1 control mAb at days 4, 6 and 8 after infection. *P* value was determined by unpaired two-tailed Student's *t* test. **(f,g)** BALB/c mice were infected with influenza and treated with IL-10R-specific mAb or rat IgG1 control mAb. At day 8 after infection, lung cells were re-stimulated with flu infected BMDC and the numbers of IFN- γ CD4⁺ **(f)** and IFN- γ CD8⁺ **(g)** T cells were quantified by ICS. *P* value was determined by unpaired two-tailed Student's *t* test. **(h)** Survival of BALB/c mice infected with influenza and treated with IL-10R-specific mAb plus vehicle control (anti-IL-10R + vehicle) or IL-10R-specific mAb plus corticosterone (anti-IL-10R + steroids). *P* value was determined by the log-rank survival test. Values are means $\pm$ s.d.

note, blockade of IL-10 action did not alter the titer of infectious virus in the lungs or virus clearance (**Fig. 5b** and **Supplementary Fig. 8** online). We next examined whether the increased mortality observed with IL-10R-specific mAb administration was due to enhanced pulmonary inflammation. To explore this mechanism, we first surveyed the BALF of infected mice that had been treated with blocking antibody or isotype control antibody for the production of a panel of inflammatory mediators. We found that expression of a number of these mediators was elevated relative to infected controls in the BALF of IL-10R-specific mAb-treated mice (**Fig. 6a** and **Supplementary Fig. 9** online).

We also surveyed potential cellular targets of IL-10 action in the lungs and found (**Supplementary Fig. 10** online) that IL-10R was preferentially expressed on inflammatory monocytic cells of the dendritic cell and macrophage lineages (**Supplementary Fig. 10**). Furthermore, we observed substantially increased numbers of the inflammatory monocytic cells infiltrating to the lungs after IL-10R blockade *in vivo* (**Fig. 6b**). IL-10R blockade also had a modest effect on neutrophil accumulation in the lungs (**Fig. 6c**). The marked increase in inflammatory monocytic cells was associated with increased production in BALF of IL-12 p40, a cytokine that is primarily produced by monocytic cells (**Fig. 6d**). IL-10R blockade also resulted in an increase in the amount of IFN- γ released into BALF (**Fig. 6e**). This increased production of IFN- γ with IL-10R blockade was associated with a modest increase in the number of virus-specific T_{eff} cells (**Fig. 6f,g**). Notably, high-dose influenza virus infection also led to a massive influx of monocytic cells and neutrophils that was associated with a decrease in IL-10 production (**Supplementary Fig. 11** online).

The above results strongly suggested that blocking of T_{eff} cell-mediated IL-10 signaling results in increased inflammation and potentially lethal injury during acute influenza infection, without affecting virus titer or clearance. Therefore, we reasoned that suppression of the exaggerated inflammatory response produced by IL-10R blockade during influenza infection may prevent the development of

lethal pulmonary injury and death. To evaluate this, we examined the effect of corticosteroid administration on the survival of infected mice undergoing IL-10R blockade. We found that corticosteroid administration partially protected infected mice from lethal injury and death (**Fig. 6h**). Of note, steroid administration at a time immediately before the influx of T_{eff} cells into the infected lungs suppressed inflammation, as monitored by the recruitment of monocytic cells to the lungs and by IL-12 p40 concentrations in BALF, but had minor effects on lung virus titers and virus clearance (**Supplementary Fig. 12** online).

DISCUSSION

In this report, we analyzed the production and function of the regulatory and anti-inflammatory cytokine, IL-10, in the respiratory tract during acute experimental influenza infection. We found that, in contrast to the persistent low level of IL-10 produced during chronic viral infection^{6–8}, acute influenza infection induces rapid and transient high-level production of IL-10 in the infected respiratory tract coincident with onset of the adaptive immune response. The source of this IL-10 is antiviral CD8⁺ and CD4⁺ T_{eff} cells themselves, with CD8⁺ T_{eff} cells probably contributing more to the total T_{eff} cell-derived IL-10 in the lungs than do CD4⁺ T_{eff} cells. We show that this T_{eff} cell-derived IL-10 has a crucial role in regulating the development of lung inflammation and lethal injury. Thus, our findings reveal a previously undescribed regulatory role for antiviral T_{eff} cells in controlling excess inflammation and associated immune-mediated pathology during acute respiratory virus infection.

Although not definitive, multiple lines of evidence reported here suggest that antiviral CD8⁺ T_{eff} cells are a major source of the IL-10 produced in the influenza-infected lungs. Several earlier reports^{18,19} as well as more recent data^{20,21} have indicated that activated CD8⁺ T cells are capable of secreting IL-10. However, it was not established whether IL-10, under these circumstances, was produced by conventional CD8⁺ T_{eff} cells or a regulatory CD8⁺ T cell subset²². Indeed, IL-10-producing CD8⁺ T cells were also detected (by ELISPOT) in lymph

nodes draining the lung²³, and IL-10 transcripts were detected in a fraction of total CD8⁺ T cells isolated from infected lungs²⁴ in experimental influenza infection. However, in these instances, it was not determined whether virus-specific CD8⁺ T cells produce IL-10 protein *in vivo* in the infected lungs. To our knowledge, this is the first report showing high-level production of IL-10 by T_{eff} cells in acute virus infection, as well as the biologically important impact of such T_{eff} cell-derived IL-10 on the outcome of infection. We speculate such a mechanism may operate in a variety of acute virus infections in which both intensive inflammation and strong T_{eff} cells responses are induced.

Consistent with the key role of CD8⁺ T_{eff} cells in virus clearance, IL-10-producing CD8⁺ T_{eff} cells are highly enriched at the site of infection. Of note, we found that the induction of these IL-10-producing CD8⁺ T_{eff} cells in the lung is dependent on the presence of CD4⁺ T cells. In agreement with previously published data^{25,26}, CD4⁺ T cells are needed neither for the accumulation of CD8⁺ T_{eff} cells nor for the production of IFN- γ and TNF- α by these cells *in vivo* during acute influenza infection (Fig. 4c and data not shown). Thus, these data raise the possibility that help from CD4⁺ T cells can shape the quality of CD8⁺ T cell responses (for example, regulatory cytokine production) during acute virus infection.

Blockade of IL-10 produced by CD8⁺ and CD4⁺ T_{eff} cells resulted in enhanced lung inflammation, elevated expression of multiple cytokines and chemokines in the infected lungs and increased mortality with no effect on virus titer or clearance. IL-10 is recognized as a regulatory (anti-inflammatory) cytokine. This cytokine can act on multiple cell types to regulate immune and inflammatory responses⁴. Inflammatory cells of the myeloid lineage, particularly the monocytic lineage (for example, inflammatory lung dendritic cells and macrophages), represent attractive and, probably, primary cellular targets for IL-10 in infected lungs. As we (Supplementary Fig. 10) and others²⁷ have shown, this cell lineage expresses IL-10R, and these cells increase in number in the infected lungs after IL-10R blockade. In this connection, it is noteworthy that a subset of monocytic cells recruited into the influenza-infected lungs has been implicated in the development of increased inflammation and associated immunopathology during experimental influenza infection²⁸. Although the mechanism of action of this T_{eff} cell-derived IL-10 has yet to be fully elucidated, IL-10-mediated inhibition of recruitment, activation and/or pro-inflammatory cytokine and chemokine production by these inflammatory monocytic cells may be essential for regulating excess inflammation during the host response to influenza infection⁴. Such an effect of IL-10 could account for the aforementioned increase in cytokine and chemokine production, as well as the increase in inflammatory cell accumulation in the infected lungs after IL-10R blockade. An associated increase in the activation state of the infiltrating inflammatory monocytic cells could also result in increased stimulation of recruited virus-specific T_{eff} cells and the increase in IFN- γ detected in the infected lungs after blockade. IL-10 may also act directly on the T_{eff} cells themselves in an autocrine fashion to dampen the response of the T_{eff} cells to viral antigen. A more complete understanding of the mechanism(s) of control of inflammation by IL-10 in this model will ultimately require the characterization of the cell types expressing IL-10R in the inflamed lungs and the response of these cell types to IL-10.

It is particularly noteworthy that blocking IL-10 activity during infection results in the overproduction of several proinflammatory mediators (for example, TNF- α , IL-6 and monocyte chemoattractant protein-1) that had been implicated in the 'cytokine storm' observed in infections with the highly pathogenic H5N1 avian and the 1918

Spanish influenza viruses^{1–3}. These highly virulent influenza strains could produce exaggerated lung inflammation and lethal illness not only by enhancing proinflammatory responses^{1–3} but also by suppressing the production of anti-inflammatory cytokines such as IL-10 by innate and, perhaps more importantly, by adaptive immune effector cells. It is further noteworthy that we saw that high-dose (lethal) influenza infection led to a disproportionate decrease in the production of IL-10 compared to IFN- γ in infected lungs. This finding raises the possibility that infection under conditions in which virus titers reach high levels in the lungs early in infection, such as is observed with highly pathogenic influenza strains^{29,30} or with high-dose infection with conventional strains³¹, may selectively suppress IL-10 production by T_{eff} cells. We also observed that corticosteroid administration after IL-10R blockade partially reversed the effect of IL-10 blockade. This is not unexpected, given the inhibitory effect of corticosteroids on the transcription of a large number of proinflammatory cytokines and chemokines, as well as of adhesion molecules and inflammatory enzymes³². This result reinforces the view that IL-10 produced by T_{eff} cells acts primarily to control the extent of inflammation induced by the adaptive (and innate) immune response during infection. Although corticosteroids can target many different cell types³², the major impact of corticosteroid treatment in this model of infection and IL-10 blockade was suppression of the expression of the inflammatory monocytic lineage cell product, IL-12 p40, again implicating a possible role of this cell lineage in the development of pulmonary inflammation and injury during infection.

In summary, our findings reveal a previously unrecognized function of T_{eff} cells, in particular CD8⁺ T_{eff} cells, in providing an essential regulatory function: control of excessive inflammation and associated tissue injury during acute viral infection. Understanding the mechanism of the expression of IL-10 by CD8⁺ T_{eff} cells and the function of this T_{eff} cell-derived anti-inflammatory cytokine may provide insight into the pathogenesis of infection with highly virulent strains of influenza as well as a framework for the design of more effective treatment modalities against these lethal infections.

METHODS

Mice and infection. We purchased wild-type BALB/c and C57/BL6 mice from Taconic Farms. We purchased *Rag1*^{−/−} mice from Jackson Laboratories. We generated IL-10-IRES-EGFP reporter mice (Vert-X mice) by insertion of a floxed neomycin-IRES-EGFP cassette between the endogenous stop site and the polyadenosine site of *Il10*. We excised the neomycin resistance marker by breeding the mice with Zp3-Cre mice, and we confirmed successful Cre-mediated deletion by Southern blot analysis (R.M. and C.L.K., unpublished data). We performed animal care and experiments in accordance with institutional and US National Institutes of Health guidelines and with the approval of the Animal Care and Use Committee of the University of Virginia. We prepared infectious stocks of mouse-adapted influenza virus A/PR/8/34 (H1N1) as previously described¹⁶. We infected 12–15 week old BALB/c mice with a dose of 500 egg infectious units of PR/8 in serum-free Iscove's medium (Invitrogen) intranasally after anesthesia with halothane.

Quantitative reverse-transcription PCR. We prepared lung single-cell suspensions as previously described¹⁶. To measure cytokine expression, we isolated RNA from magnetic cell-sorting-purified lung CD3⁺, CD3[−], CD8⁺ or CD4⁺ cells with the RNeasy kit (Qiagen) and treated it with DNase I (Invitrogen). We used oligo(dT) primers (Promega) and Superscript II (Invitrogen) to synthesize first-strand complementary DNAs from equivalent amounts of RNA from each sample. We performed real-time RT-PCR in a 7000 Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). We generated data were generated by the comparative threshold cycle (ΔC_T) method by normalizing to hypoxanthine phosphoribosyltransferase (HPRT). The sequences of primers used in the studies are available on request.

T cell re-stimulation by bone marrow–derived dendritic cells. We generated BMDCs as previously described³³. On day 6 or 7, We collected BMDCs and infected them with influenza virus at approximately 100 egg infectious units per cell for 5–6 h. Then we counted the BMDCs and mixed them with total lung, MLN or spleen cells at a 1.5 to 1 ratio in the presence of Golgi-Stop (BD Biosciences, 1 $\mu\text{l ml}^{-1}$) and human IL-2 (40 U ml^{-1}) for an additional 6 h. We performed surface staining of cell surface markers and intracellular staining of cytokines, T-bet and Foxp-3 as previously described³⁴ or according to manufacturer protocols (eBioscience). For the measuring of granule exocytosis by CD107a, we added fluorescence-labeled CD107a-specific mAb or isotype control mAb (BD Biosciences) to the *in vitro* stimulation cultures and measured the surface accumulation of CD107a by flow cytometry³⁵.

BrdU labeling *in vivo*. At day 6 after infection with influenza, we intravenously injected mice with 3 mg BrdU (BD Biosciences). Two hours later, we killed the mice and re-stimulated lung cell suspensions with influenza-infected BMDCs. We performed staining of cytokines and BrdU was performed to the manufacturer manuals.

T cell depletion *in vivo*. Three days after infection with influenza, we injected mice with 200 μg CD8-specific mAb (clone 2.43, BioExpress), 500 μg CD4-specific mAb (clone GK1.5, BioExpress) or 200 μg CD8-specific mAb plus 500 μg CD4-specific mAb intraperitoneally. We confirmed the specific depletion of the mAb by flow cytometry (data not shown).

Bronchoalveolar lavage fluid cytokine determination. We obtained BALF by flushing the airway multiple times with a single use of 500 μl sterile PBS. We spun down the BALF cells and collected supernatants for ELISA (BD Biosciences) or 23-plex cytokine array analysis (Bio-Rad) according to the manufacturer manuals.

***In vivo* intracellular cytokine synthesis assay.** We measured IL-10– and IFN- γ –producing cells *in vivo* on the basis of a previously described protocol with modifications³⁶. Briefly, at day 6 after infection, we intravenously injected mice with 500 μl of a PBS solution containing 500 μg monensin (Sigma-Aldrich) 6 h before harvesting. We prepared lung single-cell suspensions in the presence of monensin. We then fixed and permeabilized the cells and stained them for intracellular IL-10 and IFN- γ as previously described³⁴.

Interleukin-10 receptor–specific monoclonal antibody and corticosteroid administration *in vivo*. We purchased blocking IL-10R–specific mAb (clone 1B1.3A) and isotype control Rat IgG1 mAb from Bio-Express. We achieved IL-10 signaling blockade *in vivo* by injecting IL-10R–blocking mAb on day 3 (1 mg intraperitoneally in 500 μl), day 4 (0.15 mg intranasally in 40 μl) and day 6 (1 mg intraperitoneally in 500 μl). We purchased corticosterone from Sigma-Aldrich. For *in vivo* treatment with corticosterone and vehicle (10% ethanol in PBS), we intraperitoneally injected 1 mg corticosterone daily from day 5.5 to day 8.5.

Virus titer. We monitored lung viral titers via endpoint dilution assay and expressed them as tissue culture infectious dose 50 (TCID₅₀). Briefly, we incubated Madin-Darby canine kidney cells (The American Type Culture Collection) with tenfold dilutions of BALF or lung homogenate from influenza virus–infected mice in serum-free DMEM medium. After a 3-d incubation at 37 °C in a humidified atmosphere of 5% CO₂, we collected supernatants and mixed them with a half-volume of 0.5% chicken red blood cells (University of Virginia Veterinary Facilities), the agglutination pattern read, and calculated the TCID₅₀ values. For virus titrating by real-time RT-PCR, we extracted RNA from lung homogenate with Trizol (Invitrogen). We performed reverse transcription and influenza polymerase (PA) gene quantification as previously described³⁷.

FACS analysis. We purchased all FACS antibodies from BD Biosciences or eBioscience. The dilution of surface staining antibodies was 1 in 200 and the dilution of intracellular staining antibodies was 1 in 100. After antibody staining, we examined cells through a six-color FACS-Canto system (BD Biosciences). We then analyzed data by FlowJo software (TreeStar). We characterized the various cell types according to their phenotypes as follows: neutrophils (Ly6g⁺CD11b^{high}Ly6c[–]SSC^{med}), monocytic cell lineage (Ly6g[–]CD11b^{high}Ly6c⁺), natural killer cells (DX-5⁺CD3[–]), B lymphocytes

(B220⁺ forward scatter low^{low}, side scatter^{low}), CD8⁺ T lymphocytes (CD3⁺CD8⁺) and CD4⁺ T lymphocytes (CD3⁺CD4⁺).

Statistical analyses. Data are means $\pm$ s.d. We used the Kaplan-Meier log-rank survival test, one-way analysis of variance or Student's *t* test as indicated. We considered all *P* values >0.05 not to be significant.

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

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AUTHOR CONTRIBUTIONS

J.S. performed the experimental work and T.J.B. supervised the project. R.M. and C.L.K. provided Vert-X mice for the study. The manuscript was written by J.S. and T.J.B. All authors approved the final manuscript.

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