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Multiple Redundant Effector Mechanisms of CD8+ T Cells Protect against Influenza Infection

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We have previously shown that mice challenged with a lethal dose of A/Puerto Rico/8/34-OVA, are protected by injection of 4–8 × 10⁶ in vitro–generated Tc1 or Tc17 CD8+ effectors. Viral load, lung damage, and loss of lung function are all reduced after transfer. Weight loss is reduced and survival increased. We sought in this study to define the mechanism of this protection. CD8+ effectors exhibit multiple effector activities, perforin-, Fas ligand–, and TRAIL-mediated cytotoxicity, and secretion of multiple cytokines (IL-2, IL-4, IL-5, IL-9, IL-10, IL-17, IL-21, IL-22, IFN-γ, and TNF) and chemokines (CCL3, CCL4, CCL5, CXCL9, and CXCL10). Transfer of CD8+ effectors into recipients, before infection, elicits enhanced recruitment of host neutrophils, NK cells, macrophages, and B cells. All of these events have the potential to protect against viral infections. Removal of any one, however, of these potential mechanisms was without effect on protection. Even the simultaneous removal of host T cells, host B cells, and host neutrophils combined with the elimination of perforin-mediated lytic mechanisms in the donor cells failed to reduce their ability to protect. We conclude that CD8+ effector T cells can protect against the lethal effects of viral infection by means of a large number of redundant mechanisms. The Journal of Immunology, 2013, 190: 296–306.

Previous studies of the immune response to influenza infection in mice have implicated a variety of different cell types and mechanisms that collectively bring about viral clearance and provide protection. B cells can make neutralizing Ab to the coat proteins of the virus, but this occurs too late in the primary response to prevent the lethal effects of the virus and other mechanisms are needed. Studies of heterosubtypic immunity in which mice are challenged with a subtype carrying different coat proteins from the priming strain have shown that CD4+ and CD8+ T cells, nonneutralizing IgA Abs, NK cells, and γδ T cells can all contribute to heterosubtypic protection in the absence of neutralizing Abs (1). Our own studies have focused on the role of CD8 T cells and have investigated the multiple ways in which they can protect.

CD8+ T cells are cytolytic, and this is often thought of as their primary or even only role. The basic paradigms of elementary immunology tell us that B cells make Ab, T cells mediate cellular immunity, CD4+ T cells help B cells, and CD8+ T cells kill infected cells (2, 3). CD8+ T cells enjoyed a period when they were also suppressor cells and have only recently regained that reputation (4), along with CD4+ T cells. Although recognized as generalizations in need of substantial elaboration and qualification, these paradigms still restrict our thinking much more than they should.

The first modification of this picture came when it was shown that cytotoxic CD8+ T cells could also make cytokines (5, 6), and that some CD4+ T cells also can be cytotoxic (7, 8). More recently, it has been shown that CD8+ T cells can also make chemokines (9, 10), and that the interaction of CD8+ T cells with epithelial cells induced TNF secretion by the CD8+ T cells (11) and induces chemokine secretion by the epithelial cells (12). These properties lead to a whole further round of secondary effector functions, triggered originally by the CD8+ effector T cells.

As with CD4+ T cells, CD8+ T cells can differentiate along divergent lines to give rise to subsets of cells with different combinations of effector functions, and indeed, there are few functions of CD4+ T cells that cannot be carried out by CD8+ T cells and vice versa. It is even conceivable that Tc17 effectors, which lack cytolytic function, could contribute via the secretion of IL-21 to the B cell response, although this has not so far been demonstrated to our knowledge. The breadth of diversity of CD8+ T cell function has been recently illustrated by the demonstration of the very large number of products that can be produced and by the different combinations expressed by different CD8+ T cells (13).

In the model we use, polarized populations of in vitro–generated CD8+ effectors from TCR transgenic OT-1 mice, specific for the SIINFEKL peptide of OVA, are injected into naive recipients. Next, we infect the recipient mice with a genetically modified strain of influenza, bearing the SIINFEKL peptide inserted in the neuraminidase stalk. We determine the subsequent effectiveness of the injected cells in protecting the mouse from what would otherwise be a lethal challenge with the same strain of virus, using a variety of assays.

The Tc1, Tc2, and Tc17 CD8+ T cell subsets, which parallel CD4+ Th1, Th2, and Th17, have been mainly characterized by determining the phenotype of polarized subsets generated in vitro under artificial conditions (14–17), but adoptive transfer of all three subsets of effectors has been shown to protect against lethal influenza challenge.
To analyze the mechanism of protection mediated by CD8+ T cells, we have used polarized populations of in vitro–generated CD8+ effectors to dissect the role each of the subsets plays in protection. We show in this study that CD8+ T cells can contribute to and shape the immune response via a rather large number of different effector mechanisms and that, in the response to influenza, the ones highlighted in the traditional paradigm may be the least important of their accomplishments.

Cells of the Tc1 subset of CD8+ T cells can indeed kill virally infected cells by a perforin-mediated mechanism, but Tc17 cells generated in vitro initially lack lytic activity, and Tc17 prepared from perforin-deficient OT-1 are as effective at providing protection as those prepared from wild-type (WT) mice (16). Tc17 effectors, however, can kill targets in an in vivo CTL assay by a Fas ligand (Fasl)–mediated mechanism after injection into a recipient mouse. This does not appear to be essential, however, for protection against lethal influenza challenge as Fas-deficient Ipr mice are protected by Tc17 from perforin-deficient donors.

We have previously shown that Tc2 effector cells are also cytolytic (15) but are less protective against viral challenge than are Tc1. Tc2 elicit an enhanced eosinophil influx and bring about a greater impairment of lung functions (17). In the studies presented in this article, Tc17 are more effective in recruiting host CD4+ and CD8+ T cells, NK cells, eosinophils, and macrophages than Tc1.

Polarized subsets of effector CD8+ T cells (Tc1, Tc2, and Tc17) can collectively make a wide range of cytokines including IL-2, IL-4, IL-5, IL-9, IL-10, IL-17, IL-22, IFN-γ, IL-12 (9.2 U/ml, kindly provided by Stanley Wolf, Genetics Institute, Cambridge, MA), and anti–IL-4 mAb (11B11, 10 µg/ml), and anti–IFN-γ mAb (XMG1.2, 10 µg/ml) were added. For Tc1 cultures, IL-2 (4.7 µg/ml), IL-12 (9.2 U/ml, kindly provided by Stanley Wolf, Genetics Institute, Cambridge, MA), and anti–IL-4 mAb (11B11, 10 µg/ml) were added. The quality of the effector cell preparations was confirmed by phenotype analysis.

Materials and Methods

**Mice**

C57BL/6 (B6), BALB/c (BALB), B6.Thy-1.1, B6.CD45.1, B6.OT-1, B6.OT-1.Thy-1.1, B6.OT-1.CD45.1, B6.OT-1.perforin +/−, B6.1pr, B6.TCR b6−/−, B6.RAG-2 −/−, and clone 4 (BALB.HA) mice were bred at the Trudeau Institute and at University of Massachusetts Medical School (Worcester, MA), and were used at 5–8 wk of age for generation of effectors and at 8–12 wk of age for recipients. B6.OT-1.TRAIL −/− mice were kindly supplied by T.S. Griffiths (University of Iowa, Iowa City, IA); B6.1pr were purchased from Jackson Laboratories. All animal procedures were approved by the Institute Animal Care and Use Committee at the Trudeau Institute and University of Massachusetts Medical School.

**Influenza virus, infections**

Influenza A/Puerto Rico 8/34 (PR8) and PR8-OVA δ (kindly provided by Dr. Richard Webby, St. Jude Children’s Research Hospital, Memphis, TN) were grown in the allantoic cavity of embryonated hen eggs from virus stocks. Lightly anesthetized mice were infected with influenza by intranasal (i.n.) inoculation of 50 µl virus in PBS. For the stock of PR8 used, 2 × 106 EID50 = 4 LD50 and 970 PFU = ~2 LD50 for PR8-OVA δ, PR8 titers

Mice injected with CD8+ T cells and influenza infected were euthanized at various times postinfection by cervical dislocation. The lungs were removed, teased into single-cell suspensions in a fixed volume of 5 ml, and then 1 ml aliquots frozen and stored at −70°C. The lysates were thawed and the influenza titer determined using the Madine-Darby canine kidney cell plaque assay as detailed previously (15). Results are expressed as PFUs per lung. In some experiments, viral titer was determined by RT-PCR (see below).

**Generation of Tc1 and Tc17 CD8+ effector cells in vitro**

To analyze the mechanism of protection mediated by CD8+ T cells act by multiple redundant protective mechanisms. We have previously shown that Tc2 effector cells are also cytolytic (10, 15). We show in this article that these mediators recruit B cells to the lung. We show also that the CD8+ T cells can indeed kill virally infected cells by a perforin-mediated mechanism, but Tc17 cells generated in vitro initially lack lytic activity, and Tc17 prepared from perforin-deficient OT-1 are as effective at providing protection as those prepared from wild-type (WT) mice (16). Tc17 effectors, however, can kill targets in an in vivo CTL assay by a Fas ligand (Fasl)–mediated mechanism after injection into a recipient mouse. This does not appear to be essential, however, for protection against lethal influenza challenge as Fas-deficient Ipr mice are protected by Tc17 from perforin-deficient donors.

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Polarized subsets of effector CD8+ T cells (Tc1, Tc2, and Tc17) can collectively make a wide range of cytokines including IL-2, IL-4, IL-5, IL-9, IL-10, IL-17, IL-22, IFN-γ, IL-12 (9.2 U/ml, kindly provided by Stanley Wolf, Genetics Institute, Cambridge, MA), and anti–IL-4 mAb (11B11, 10 µg/ml), and anti–IFN-γ mAb (XMG1.2, 10 µg/ml) were added. For Tc1 cultures, IL-2 (4.7 µg/ml), IL-12 (9.2 U/ml, kindly provided by Stanley Wolf, Genetics Institute, Cambridge, MA), and anti–IL-4 mAb (11B11, 10 µg/ml) were added. The quality of the effector cell preparations was confirmed by phenotype analysis.

**Phenotype of Tc effectors**

Tc effectors were prepared as described earlier. For intracellular cytokine staining, single Tc effector cell suspensions were cultured for 4 h with 10 ng/µl PMA, 500 ng/ml ionomycin, and 10 ng/ml brefeldin A. Cells were harvested and incubated with Ab to cell surface markers and then fixed with fixation/permeabilization solution (BD Biosciences) for 10 min. Cells were then washed with 1X Perm/Wash buffer (BD Biosciences) and then permeabilized with 1X Perm/Wash buffer with 0.05% Triton X-100 for 10 min. Then cells were incubated with Abs to cytokines in 1X Perm/Wash buffer with 0.05% Triton X-100, 1L-17–PE and IFN-γ–FITC (XMG1.2; BD Biosciences). Cells were analyzed on the CyA LX9 laser flow cytometer (DAKO), the BD FACS Canto, or on the BD LSRII. The staining profiles were analyzed using FlowJo.

**In vivo cytotoxicity**

In vivo cytotoxicity was assayad as follows: Tc1 and Tc17 effector populations were prepared from OT-1 mice (CD45.2 Thy1.2+), and 4 × 106 cells of Tc1 or Tc17 were injected into naive B6.Thy1.2+ recipients (CD45.2+ Thy1.1+). One day after Tc effector injection, recipient mice were injected with 2.5 × 105 SFINFELK–pulsed spleen cells stained with 1.25 µM CFSE (CD45.1+ SFSE δ), and 2.5 × 105 of nonpulsed spleen cells stained with 156 nM CFSE (CD45.1+ CFSE δ). Twenty-four hours later, spleen cells were harvested and the ratio of surviving CD45.1+ CFSE δ to CFSE δ cells was measured by flow cytometry.

For Fig. 1B, Tc1 and Tc17 effectors were prepared from WT OT-1 (CD45.1+ CD45.2+), and 4 × 106 cells of Tc17 or Tc1 were injected into naïve B6.CD45.1 recipients (CD45.1+). One day after Tc effector injection, recipient mice were injected with 1.25 × 105 SFINFELK–pulsed WT cells (CD45.2+ CFSE δ) and 1.25 × 105 nonpulsed WT cells (CD45.2+ CFSE δ) or 1.25 × 105 SFINFELK–pulsed Fas mutant (ipr) target cells (CD45.2+ CFSE δ and 1.25 × 106 nonpulsed ipr lpr cells (CD45.2+ CFSE δ). Twenty-four hours after transferring target cells, mice were sacrificed and spleen cells were isolated. Percentage killing was calculated by comparing the ratio of surviving CFSE δ targets to CFSE δ targets in spleen.

For the ex vivo CTL assay, Tc1 and Tc17 effectors were prepared from OT-1 (CD45.2+), and 4 × 106 cells of Tc17 or Tc1 were injected into naïve B6.CD90.1 recipients. Two days after Tc effector transfer, recipient mice were injected with 0.2 LD50 of PR8-OVA δ (1,200 diluted). On day 5 of infection, transferred cells were purified from the lung by cell sorting, and 2 × 106 cells of purified Tc17 or Tc1 were injected into naïve B6.CD45.2 mice. One day after transfer, recipient mice were injected with 2 × 106
(1:1) or 2 × 10^5 (1:0.1) of S1NFEK1-pulsed cells (CFSE™) and nonpulsed cells (CFSE™). Twenty-four hours after CFSE-stained cell transfer, the ratio of surviving CFSEplus cells to CFSEminus cells was determined by flow cytometry.

Adaptive transfers, lethal infection, weight changes, and survival

B6 mice were injected i.v. with 4, 8, or 16 × 10^5 OT-1 Tc17 or OT-1 Tc1 effector cells on day −1 and challenged on day 0 with an i.n. lethal dose of 1–2 LD50 influenza PR8-OVA virus or 1–3 LD50 influenza PR8 virus. In other experiments, polyclonal CD8 effectors specific for PR8 Ags were isolated from mice 7 d after sublethal viral challenge and used as the donor cells. Mice were weighed every second day and weight expressed as percentage of initial. A cohort of mice was followed up to days 12–28 post-challenge to determine percentage survival. In some experiments (Fig. 8), hemagglutinin (HA)-specific clone 4 TcR transgenic mice were used as donors and BALB/c as recipients.

Adaptive transfers, sublethal infection, albumin and lactate dehydrogenase measurement, and lung function

B6.OT-1 effectors were injected into B6.CD45.1 mice. One day later, mice were infected i.n. and then sacrificed at various time points after infection (days 2, 4, 6, 8, and 21 postinfection). Lungs were removed after perfusion with 5 ml PBS via the left ventricle of the heart, and single-cell suspensions were prepared by collagenase treatment (5 mg/ml collagenase A and DNase I). Cells were stained with anti-CD45.2 to distinguish donor and host cells, and surface markers were stained with the following Abs: CD45.2-AlexaFluor647, CD4-PE, CD8-PE-Cy7, CD19-FITC, and AQUA fixable Dead Cell Stain (Invitrogen). Cells were analyzed on the FACS Canto II (BD). Cells were gated on live (AQUAnegative) and either CD45.2+ (host) or CD45.2− (donor) and analyzed using FlowJo.

Neutrophils were identified as Gr1highCD11bhighF4/80+I-Ab−; NK cells were identified as gal-ser/mCD1d+ (PBS-57 tetramer; National Institutes of Health Tetramer Core). Biotinylated Abs were counterstained with streptavidin-Pacific orange (8 mg/ml; Invitrogen) and then fixed with 4% paraformaldehyde unless otherwise noted. RNA was extracted and purified from CD8+ T effector cells, using TRIzol (Invitrogen) and RNeasy kit (Qiagen, La Jolla, CA), sequentially. DNase-treated RNA (2 μg) was reverse transcribed with Oligo dT and SuperScript II (Invitrogen). Quantitative PCR was performed using TaqMan Universal PCR Master Mix, following the Applied Biosystems (Foster City, CA) protocol. Primers and probes for GAPDH, FasL, and TRAIL were obtained from Applied Biosystems. Quantitative PCR was performed using a PRISM 7700 instrument (Applied Biosystems). Quantitation of viral RNA was performed as previously described (18) using forward (5'-GAGGAGTTGAG-3') and reverse (5'-TCATCACCCTAGGA-3') primers that were designed for a viral acidic polymerase fragment.

Cytokine assay

Levels of cytokines and chemokines in lung homogenates (Fig. 8) were determined using mouse multiplex Luminex kits (Inovitrogen) read on a Luminex 100 reader (Luminex).

Staining lung sections

Infected mice were sacrificed and bled by cutting the renal artery. Lungs were perfused in 4% paraformaldehyde and embedded in paraffin. Five-micrometer paraffin lung sections were cut in a microtome and collected in plus slides. Slides with lung sections (for Supplemental Fig. 3) were incubated in a 60°C oven and quickly transferred to xylene. Lung tissues were progressively hydrated by transferring them to xylenes, alcohol, 96% alcohol, 70% alcohol, and finally water. Aqs were unmasked by boiling lung sections in Ag retrieval solution for 30 min (Dako). Slides were cooled down for 20 min and washed with deionized water. Lung sections were outlined with a pap-pen and blocked for 30 min with 5% normal donkey serum and 1:100 of Abs against FcRs 2.4G2 diluted in 0.1% Tween 200/1% Triton X-100 in PBS. Without washing, primary Abs (Santa Cruz Biotechnology) clone M-20; proliferating cell nuclear Ag (Santa Cruz Biotechnology) clone c-20 and biotinylated B220 (BD Pharmingen, RA36B2) were added to the lung sections and incubated overnight at room temperature in a humid chamber. CD3 and proliferating cell nuclear Ag were detected with donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 594 (Molecular Probes, Eugene, OR), and B220-biotin was detected by adding donkey anti-rat (Jackson ImmunoResearch Laboratories), Alexa Fluor 488 ( Molecular Probes), and streptavidin, Alexa Fluor 488 (Molecular Probes). In other experiments, lung sections were stained with anti-prosurfactant protein C as an indicator of type II epithelial cells, or cell suspensions were prepared an analyzed by flow cytometry using the same reagents. Tissue sections were mounted with medium for fluorescence with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Pictures were taken with a Carl Zeiss Microscope, and representative ×200 magnifications are shown.

Results

In our previous studies (15–17), we have seen evidence of the participation of IFN-γ, IL-4, and IL-17-secreting cells in the response to influenza, suggesting that Tc1, Tc2, and Tc17 cells can protect naive mice from otherwise lethal challenge, but our previous studies had measured only weight changes and survival. We show in this study that injection of Tc1 or Tc17 effectors reduces viral load and lung pathology. We have previously shown (15–17) that all three subsets of cells can protect naive mice from otherwise lethal challenge, but our previous studies had measured only weight changes and survival. We show in this study that injection of Tc1 or Tc17 effectors reduces initial viral load (Supplemental Fig. 1A), prevents damage as shown by reduction in leakage of albumin (Supplemental Fig. 1B) and lactate dehydrogenase (Supplemental Fig. 1C) into the lung lavage, and alleviates respiratory distress as shown by changes in minute volume (Supplemental Fig. 1D) and respiratory rate (Supplemental Fig. 1E).

Adaptive transfer of CD8+ effectors brings about a reduction in viral load and lung pathology

In vivo–generated polyclonal CD8+ effectors also provide protection

It is not possible to prepare well-polarized CD8 effectors in vivo, but polyclonal CD8 effectors were isolated from mice challenged with 0.5 LD PR8 7 d postinfection, and graded numbers were injected into naive recipients that were then challenged with 3 LD PR8. As few as 4 × 10^6 effectors reduced weight loss and increased survival, establishing that protection was not unique to the use of TcR transgenic T cells (data not shown).
**CD8\(^*\) effectors that lack perforin-mediated cytotoxic activity are still protective**

The prevailing belief is that CD8\(^*\) T cells protect against viral infection by killing virally infected cells. We have previously shown that Tc1 and Tc17 effectors are equally protective even though Tc1 are lytic in vitro, whereas Tc17 are not (16). We showed also that protection by Tc1 was diminished in effectors made from perforin\(^{-/-}\) mice, whereas protection by Tc17 was not (16). Although Tc17 effectors lacked FasL or TRAIL expression in vitro (Supplemental Fig. 2), it remained possible that Tc17 develop in vivo lytic activity dependent on FasL or TRAIL expression on injection back into the animal. To examine this possibility, we injected Tc17 effectors from OT-1 mice into uninfected naive recipients and looked for killing of SIINFEKL-labeled targets at 24 h in an in vivo cytolysis assay.

When Tc17 effector cells are injected into normal mice, they do show some killing activity at 24 h after injection, but less activity than Tc1 (Fig. 1A). The Tc17-mediated killing, however, is FasL dependent because Fas-negative targets from lpr mice are not killed (Fig. 1B). Eventually, however, Tc17 cytolytic activity develops and becomes quantitatively equivalent to that of Tc1 by day 5 (Fig. 1C), and many of the injected cells become double producers of IFN-\(\gamma\) and IL-17 (data not shown).

To determine whether FasL-mediated killing plays a role for Tc17-mediated protection, we injected Tc1 or Tc17 effectors from WT or perforin-deficient mice into WT or lpr recipients to determine whether they could still protect in the absence of both perforin and FasL-mediated killing. WT Tc17, WT Tc1, and perforin-deficient Tc17 were fully protective in WT recipients (Fig. 1D), but mice that received Tc1 from perforin-deficient mice lost more weight (Fig. 1D) and two of four died. This differential was more marked when the same cells were transferred into lpr recipients where Tc1 cells from perforin-deficient mice provided no protection, whereas Tc17 WT or perforin-deficient effectors were still protective (Fig. 1E). We conclude that although perforin-mediated lysis is important in the protection mediated by Tc1 and Tc2 cells, neither perforin nor FasL-mediated killing play a significant role in the protection mediated by Tc17.

In further experiments, we attempted to determine whether Tc1 or Tc17 protection is mediated by a TRAIL-dependent mechanism using Tc1 and Tc17 effectors prepared from CD8\(^*\) T cells from OT-1.TRAIL\(^{-/-}\) mice. Tc17 effectors from TRAIL-deficient mice were still able to protect (data not shown), but a high proportion of the CD8\(^*\) cells in the naïve OT-1.TRAIL\(^{-/-}\) mice were CD44\(^{hi}\), and it was not possible to make preparations of Tc17 effectors from these mice with anything more than a very low percentage of IL-17-secreting cells. We were thus unable to completely exclude the possibility that Tc17 effectors protect by a TRAIL-mediated lytic mechanism, as suggested by Brincks and colleagues (19). We turned next to other correlates associated with the protection mediated by the injection of CD8\(^*\) effectors to determine whether any were essential for protection.

**Adoptive transfer of CD8 effectors enhances recruitment of host cells**

CD4\(^*\) and CD8\(^*\) T cells, B cells, neutrophils, NK cells, and macrophages are all recruited into the lungs of influenza-infected mice (20, 21). The injection of already functional CD8\(^*\) effectors from OT-1 mice accelerates the recruitment of most of these cell types after exposure to PR8-OVA\(_2\). Differential effects are seen with effectors of different subsets; thus, Tc2, for example, recruit greater numbers of eosinophils than Tc1 (17), and Tc17 recruit more B cells (see later). We had also previously shown that the injection of Tc17 effectors led to a greater accumulation of neutrophils than Tc1 after viral challenge (16). In further experiments, we found that after the adoptive transfer of either Tc1 or Tc17 effectors, Tc17 effectors recruited greater numbers of cells to the lung than Tc1 recipient or control mice (Fig. 2A), and that Tc17 cells entered the lung more rapidly and in greater numbers than Tc1 cells (Fig. 2B). Tc17 effectors were able to recruit host CD4\(^*\) (Fig. 2C) and CD8\(^*\) T cells (Fig. 2D) more effectively than Tc1 effectors. However, host CD4\(^*\) T cells rapidly declined in the recipients of CD8\(^*\) effectors after 20 d, whereas they continued to increase in the untreated controls, and the net effect of the transfer was actually to decrease recruitment of host T cells at later time points. There was, however, a striking increase in the number of B cells (Fig. 2E) after adoptive transfer of Tc17, which persisted through day 20; clusters of B cells were seen in the lungs of mice 8 d after transfer of Tc17 cells (Supplemental Fig. 3). We concluded that the cytokines and chemokines released after the transfer of Tc17 and, to a lesser extent, Tc1 led to an early enhancement of recruitment of many cell types, including neutrophils, NK cells, mac-
cells (recruited into the lungs. Similar results were seen in two experiments. Injected with $8 \times 10^6$ of CD8 effector T cells and viral challenge. Groups of five B6 mice were protected from lethal challenge by the transfer of Tc1 rather than Tc17. Additional mice from each protocol were run in parallel, receiving Tc17 versus none, and uninfected versus none, all p $< 0.05$.

FIGURE 3. Accelerated regeneration of type II epithelial cells in protected mice. (A) Groups of five B6 mice were injected with $8 \times 10^6$ in vitro–generated Tc1 (●) or Tc17 Thy-1.1.OT-1 effectors (■) or left uninjected (▲) and challenged 1 d later with 0.2 LD$_{50}$ PR8-OVA1. Mice were sacrificed at the times indicated, and lung sections were prepared and stained with fluorescently labeled Ab to prosurfactant protein C as a marker for type II epithelial cells to determine the numbers of positive cells per field. For the differences between Tc17 versus no transfer at days 6 and 8, p $< 0.001$, for Tc1 versus no transfer p $< 0.01$, and for Tc17 versus Tc1, p $< 0.01$. (B) Cell suspensions from the lungs of similarly treated mice were prepared from mice sacrificed at day 8 and analyzed with the same fluorescent-labeled Ab by flow cytometry. Tc17 versus none, Tc1 versus none, and uninfected versus none, all *p $< 0.05$.

Protection is still seen when host neutrophils are depleted

Neutrophils are generally thought to exacerbate immunopathology in viral infections, but we had seen an early spike in neutrophil numbers in studies of heterosubtypic protection (data not shown) that was accompanied by an early reduction of the viral load. We speculated that an early, moderate influx of neutrophils might be beneficial to the outcome of the infection, whereas the damage done by larger numbers at later times was a correlate of the failure to control infection.

Two groups of naive B6 mice were injected with $8 \times 10^6$ Tc17 effectors at day zero and infected with 2 LD$_{50}$ PR8-OVA1 to test the role of neutrophils in infection. One group of five mice was injected with 200 μg of the mAb, 1A8, to deplete the Ly6G-high neutrophils on days −1, +1, and +3, whereas the second group was injected with an equivalent amount of isotype control Ig. Two additional groups of mice were run in parallel, receiving Tc1 rather than Tc17. Additional mice from each protocol were sacrificed to check for neutrophil depletion as judged by staining with fluorescently labeled anti–GR-1 (RB6) Ab. A fifth group of mice received no CD8* effectors and no Ab, but were challenged with virus. Weight loss and survival were followed for 28 d. No effects were observed on either weight changes or survival (Fig. 4A) after effective neutrophil depletion (Fig. 4B). We concluded that neutrophil recruitment does not play an essential role in protection.

Protection is still seen when host NK cells are depleted

A similar experiment was carried out to determine whether NK cell depletion, using 200 μg NK-1.1 Ab, injected on days −1, +2, and +5, would affect the level of protection. Again, there was no effect on either weight changes or survival (Fig. 4C) after effective NK cell depletion (Fig. 4D). We concluded that NK cell recruitment does not play an essential role in protection.
FIGURE 4. Depletion of host neutrophils or NK cells does not diminish protection. A total of $8 \times 10^6$ in vitro-generated Tc1 or Tc17 OT-1 effectors were injected into groups of five naive B6 recipients. Mice were challenged with 2 LD$_{50}$ PR8-OVA$_{3}$ (A). Mice were depleted of neutrophils or received isotype control Ig, and weight changes and survival were tracked for 30 d. The effectiveness of the neutrophil depletion is shown in (B). (C) Mice were depleted of NK cells or received isotype control Ig, and weight changes and survival were tracked for 13 d. The effectiveness of the NK cell depletion is shown in (D). The details of all of the procedures are as described in Materials and Methods. Similar results were seen in two experiments.

**Protection is still seen when host T cells are absent**

Although adoptive transfer actually decreased the recruitment of host T cells at later time points, we considered it possible that they might still play some role in the early clearance of virus. We therefore determined the ability of Tc1 and Tc17 effectors to protect mice lacking both CD4$^+$ and CD8$^+$ T cells. For this we used TCR$^b$/TCR$^d$-deficient recipient mice. Uninjected $\beta^d$ mice lost weight rapidly, and all died by day 10 when challenged with 3 LD$_{50}$ PR8-OVA$_3$ (Fig. 5A). $\beta^d$ mice injected with either $8 \times 10^6$ Tc17 or Tc1 effectors at day −1 started to regain weight by day 5 and were fully recovered by day 10. Individual mice began to lose weight again around day 30 and eventually died (data not shown), possibly after development of viral escape mutants, although this was not analyzed. We concluded that neither recruited host CD4$^+$ nor CD8$^+$ T cells are crucial for early protection.

**Protection is still seen when host T cells and B cells are absent**

Finally, we examined whether protection could be seen in the absence of both $\alpha\beta$ and $\gamma\delta$ T cells and B cells. A total of $8 \times 10^6$ Tc1 or Tc17 effectors were injected into RAG-2$^{-/-}$ host, which were challenged with 3 LD$_{50}$ PR8-OVA$_3$. Yet again, the injected effectors were able to reverse weight loss and protect the mice for at least 15 d as shown in Fig. 5B. As with the $\beta^d$-deficient recipient mice, individual mice started to die at later time points (data not shown), but, again, it was clear that none of absent cell types was required to generate the initial protection.

**Protection is still seen when multiple cell lineages are absent or depleted and donors are perforin deficient**

In a further attempt to show clusters of mechanisms that, collectively, were essential for protection, we engineered multiple deficiencies into a single protocol. Tc1 (Fig. 6A, 6B) or Tc17 (Fig. 6C, 6D) effectors were prepared from CD8$^+$ and CD8$^+$ T cells, B cells, and neutrophils; in addition, the donor cells were deprived of perforin-mediated killing. Under these conditions, the Tc17 perforin-deficient effectors were still able to protect RAG-2-deficient recipients from lethal challenge, even when neutrophils were depleted (Fig. 6C, 6D). Tc1 effectors from perforin-deficient donors, however, were less protective than Tc1 effectors from WT mice in RAG-2$^{-/-}$ hosts, again exposing a difference in the mechanism of protection by Tc17 and Tc1 effectors.

We were somewhat surprised at the robustness of the protective effect thus revealed, and next attempted to determine which parts of the protective effect were Ag specific and which were Ag nonspecific. We carried out experiments in two models: in the first, to determine how long the donor cells needed to be present in the recipient after transfer to retain protection; and in the second,
The efficacy of the donor cell depletion by injection of anti-Thy-1.1 is illustrated in Fig. 7C, which shows that the percentage of Thy-1.2 + CD8 + donor cells was reduced from 22.5 to 0.52.

Note that all of the weight change data presented in Fig. 7A and 7B are from a single experiment but are separated into different panels so that the weight loss curves may be distinguished. This pattern of results was seen in each of two experiments and suggests that an initial period that requires the continued presence of the donor cells is followed by, at day 5, a period in which the original injected cells are no longer required. This implies that the CD8 effectors had set in motion a train of protective events that no longer required their presence. The requirement for the persistence of Tc1 effectors would appear to be more prolonged for Tc17-mediated protection.

The OT-1 response to the OVA-bearing virus (PR8-OVA1) provides bystander protection against the virus lacking the OVA epitope (A/PR8)

Mice were injected with Tc1 (Fig. 8A) or Tc17 (Fig. 8B) effectors generated from OT-1 mice and were challenged with ~1 LD50 of the “specific” virus, PR8-OVA1, or a lethal dose of the nonspecific virus, 4 LD50 PR8, or both.

All the mice receiving Tc1 effectors from OVA peptide-specific OT-1 mice died when challenged with PR8 alone, whereas one of four survived when challenged with both viruses and regained weight (Fig. 8A). All mice challenged with 1 LD50 PR8-OVA1 lost less weight, recovered, and survived. The difference was more marked when mice received Tc17 effectors (Fig. 8B). Three of four mice challenged with PR8 lost weight rapidly and died. Mice challenged with both viruses also lost weight as rapidly as those challenged with PR8 alone but started to recover at day 10, and three of four surviving, suggested that the addition of the specific virus led to protection against the nonspecific virus. Only one of four mice survived PR8 alone. A similar pattern of reduced weight loss when both viruses were given was seen in a second experiment in which a lower challenge dose of PR8 was used. In a further experiment, we used LPS-free OVA instead of PR8-OVA1 and again observed a bystander effect with protection against a lethal challenge with PR8 (Fig. 8C). This eliminates the possibility that the apparent bystander effect was really because SIINFEKL-specific T cells killed doubly infected cells and the possibility that the two viruses in some way compete with one another. There was a small reduction in viral load at day 3 in mice given PR8 plus OVA compared with PR8 alone, but it was not sustained and the titer had rebounded by day 7 (Supplemental Fig. 4).

We conclude that, together, the effector depletion and the bystander protection experiments support a model in which an Ag-specific step leads to a subsequent protective mechanism that is no longer Ag specific.

The transfer of CD8 + rested effectors leads to activation of an early host innate response

We have previously shown that the adoptive transfer of memory CD4 + cells can bring about an induction of a number of innate cytokines and chemokines early in the response to influenza infection (22), and that this was correlated with a 10-fold reduction in viral titer and an increase in survival. CD8 + effectors express many of the same effector mechanisms expressed in CD4 +, and it seemed possible that they too might elicit early innate responses.

We found, in this study, that the adoptive transfer of rested CD8 + effectors from the HA-specific TcR transgenic clone 4 BALB/c mice were also able to induce this same early response (Fig. 9). In this experiment, we used effectors that were rested 3 d before transfer. Such cells adopt a memory phenotype (23) but become...
reactivated to effectors on transfer to infected mice (24). The rested CD8\(^+\) effectors were transferred into naive BALB/c recipients, which were then challenged with 2 LD\(_{50}\) PR8. The mice were sacrificed at days 1, 2, or 3, and the supernatant from the lung homogenates assayed for a panel of cytokines and chemokines using Luminex. Mice injected with memory CD4 T cells show enhanced survival (25) after viral challenge, and memory CD8 are also effective (data not shown). The factors indicated were in-
Challenged with 2 LD50 PR8. Mice were sacrificed on day 1, 2, or 3, and the supernatants of lung homogenates assayed for the cytokines and chemokines.

Number of CD8+ effector T cells generated in vitro, from SIIN-FEKL/Kb-specific TCR transgenic mice. A number of investigators, including us (26), have shown that the adoptive transfer of large numbers of naive transgenic cells is both unnecessary and unphysiological, and that the expansion of such cells in response to challenge is inversely proportional to the input number. We argue, however, that the transfer of large numbers of effector cells is needed to mimic the normal response. We have previously shown (15) that after the adoptive transfer of large numbers of effector cells, significant numbers of the donor cells could be detected in the lung and bronchoalveolar lavage by day 1, and that several million donor cells could be found at days 3 and 5. This number is approximately equal to the number of polyclonal CD8+ T cells seen at the peak of the response of a normal mouse to infection with 1 LD50 PR8 in the absence of any transferred cells, as shown in our previous publications (16, 21). Effectors whether generated at the peak of the response or adoptively transferred produce very high levels of cytokines, and these can be expected to play a major role in protection.

All three subsets of CD8 effectors (Tc1, Tc2, and Tc17) were separately able to provide effective protection against a lethal dose of influenza virus when 4–8 million cells were transferred (15–17, this article). It is clear, in the experiments presented in this article, that the adoptive transfer of either Tc1 or Tc17 effectors brings about a large number of potentially protective changes in the host. This was also true for Tc2, as shown in earlier studies (15, 17). We showed, for both Tc1 and Tc17, that donor cells enter the lung and secrete an assortment of cytokines and chemokines (10, this article). Further, host B cells, neutrophils, NK cells, and macrophages are all recruited in large numbers after adoptive transfer of CD8+ effectors. Tc17 cells are more effective than Tc1 in this regard. Tc17 also recruit higher numbers of host CD4+ and CD8+ T cells, but the numbers recruited are actually less than in the untreated mice. It seems that recruitment stops as soon as the virus is cleared in the treated mice, whereas the influx of T cells continues in the untreated mice and reaches much higher levels. We also found some evidence for accelerated regeneration of type II epithelial cells (Fig. 3) and accelerated Ab responses (data not shown), but did not establish whether this was the cause of protection or the consequence of the mice surviving, and thus being able to recover. These are all potential candidates for bringing about viral clearance and protection against influenza.

We had initially sought to identify the essential mode of protection of CD8+ effectors by the deletion of some element that would diminish or eliminate the protective effect of the adoptive transfer. Our first thought was that protection would be dependent on the cytolytic activity of the CD8+ effectors, and it was therefore surprising that Tc17 effectors that lacked lytic activity when prepared in vitro were as effective as the lytic Tc1 effectors. We found that Tc17 regained some slight activity on introduction into a normal recipient and eventually became as lytic as the reinjected Tc1. We found, however, that the protective activity was still present in Tc17 effectors obtained from perforin-deficient mice, showing that perforin-mediated lytic activity was not essential for Tc17 effectors. Further experiments demonstrated that Tc17 cells were still protective in the absence of both perforin- and FasL-mediated lysis. We could find no evidence that protection was TRAIL mediated, but preparations of Tc17 effectors that we generated from the TRAIL−/− mice had much lower numbers of IL-17-secreting cells than the other Tc17 preparations from WT or perforin-deficient mice.

**FIGURE 9.** CD8 rested effector responses in the lung acutely enhance a broad panel of cytokine and chemokines. Rested effectors were prepared by 4-d in vitro stimulation of CD8 cells from clone 4 mice bearing the TCRs for the EYSTVASSL peptide from the HA of PR8. The effectors were then washed and cultured in the absence of cytokines for a further 3 d. A total of 5 × 10^6 naive or CD8 rested effectors were injected into naive BALB/c mice, then challenged with 2 LD50 PR8. Mice were sacrificed on day 1, 2, or 3, and the supernatants of lung homogenates assayed for the cytokines and chemokines indicated by Luminex. Similar results were seen in two experiments.
Depletion of neutrophils or NK cells was also without effect, showing that these cell types were not essential for early protection. Protection was also seen in the absence of host CD4⁺ and CD8⁺ T cells, and even in the absence of all T and B cells. In our earlier study (16), we had shown that the absence of perforin-mediated cytolytic activity was without effect on the protective activity of Tc17 but led to some loss of protection by Tc1 cells. We had also shown that Tc17 cells from IFN-γ-deficient mice were somewhat less effective in preventing reduction in their ability to reduce weight loss and improve survival (16). We did not establish any mechanism for the reduced efficiency of the deficient cells in this study, but in an earlier study, we showed that IFN-γ was important in recruiting donor cells into lungs (17) and into tumors in a model in which Tc1 effectors from OT-1 mice rejected OVA-secreting EG7 intradernal tumors (27). Titration of the IFN-γ-deficient donor cells showed that higher numbers of donor cells were able to provide full protection.

The elimination of potential protective mechanisms two at a time, three at a time, four at a time, or even at set of five at a time had little, if any, effect on the degree of protection by Tc17 but revealed some dependence on perforin by Tc1. It is important to note that the relative importance of different mechanisms is probably different in the various protocols we used; thus, in the absence of host T and B cells, perforin-mediated killing by the Tc1 donor cells begins to be of some importance, whereas in the presence of host T and B cells, the removal of perforin has no effect. It was beyond our resources to titrate the number of CD8 effectors to provide bystander protection to the PR8, suggesting that an initial Ag-specific phase was followed by a phase in which protection was mediated by some nonspecific mechanism. This latter effect was more pronounced with Tc17 effectors than with Tc1.

Because of the extreme redundancy of the mechanisms of protection, none of them was essential. The induction of host innate cytokines and chemokines as shown in Fig. 9 was striking, but we were not able to design an experiment in which only this mechanism is disabled to test how much protection was dependent on this effect. We had previously seen that innate cytokines are strongly induced at 48 h after heterosubtypic challenge (S. Misra, R.W. Dutton, T.M. Strutt, and K.K. McKinstry, unpublished observations), and others have shown that stimulation with TLR ligands can provide some level of protection in a number of models. It is thus possible that the induction of host innate cytokines and chemokines as shown in Fig. 9 is of critical importance, and further studies will be required to determine whether it is essential for protection.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure Legends:

Suppl. Figure 1 Adoptive transfer of CD8 effectors brings about a reduction in viral titer, a decrease in lung damage, and an increase in lung function. B6 mice were injected with 8 x10^6 \textit{in vitro} generated Tc1 or Tc17 OT-1 effectors or left uninjected and challenged one day later with approximately 0.2LD_{50} PR8-OVA_{1}. Some mice were sacrificed at day 3 and 7 and the lungs assayed for viral titer (panel A), others were sacrificed at the time indicated, the BAL harvested and assayed for albumin (panel B) or Lactate dehydrogenase (LDH) (panel C). Yet other mice were placed in a Buxco Plethysmograph to measure the minute volume (panel D) and the respiratory rate (panel E). Similar results were seen in two experiments.

Suppl. Figure 2 Tc17 effectors lack FasL or TRAIL expression \textit{in vitro}. Tc1, Tc2 and Tc17 effectors were prepared and RNA message levels for FasL and TRAIL (product of \textit{ttnsf10}) determined as described in the Materials and Methods. Similar results were seen in two experiments.

Suppl. Figure 3 Adoptive transfer of Tc17 effectors enhances the development of B cell clusters in lung following viral challenge. 8 x10^6 \textit{in vitro} generated Tc17 OT-1 effectors were injected into groups of 5 naïve B6 recipients which were challenged with 2LD_{50} PR8-OVA_{1}. Mice were sacrificed at day 4 and day 7. Lungs were perfused with 4% PFA and embedded in paraffin. 5 μm thick, formalin-fixed, paraffin lung sections were probed with antibodies against CD3 and B220. CD3^+ cells are stained in red and B220 lymphocytes in green (panel A). A combination of antibodies against proliferating cell nuclear antigen (PCNA) and B220 was used to detect proliferating lymphocytes (PCNA^+B220^-) and B cells (PCNA^+B220^+). Nuclear red stain labels proliferating cells and green membrane stain identifies B cells (panel B). Representative pictures from a single experiment are shown, 200x magnification.
Suppl. Figure 4. Bystander protection is accompanied by a transient lowering of viral load. As in Figure 8, 8 x10^6 in vitro generated Tc1 (panel A) or Tc17 OT-1 (panel B) effectors were each injected into three groups of mice. One was challenged with 1LD_{50} A/PR8 a second with 1LD_{50} PR8-OVA\textsubscript{1} and the third with both viruses (n=4). In panel C, the third group of mice was injected with Tc1 effectors and challenged i.n. with 100 μg LPS free ova instead of A/PR8-OVA\textsubscript{1} (n=5). Mice from each group were sacrificed at days 3 and 7 and the lungs removed for the assay of viral titer by RT-PCR.
Sup Figure 2

A  

B
Sup Figure 3

A

Day 4 post Tc17 transfer and infection

B

Day 8 post Tc17 transfer and infection
Sup Figure 4

![Graph showing PA copies per lung (Log_{10}) over days 3 and 7 for different treatments.]

- Tc1: PR8-OVA
- Tc1: PR8
- Tc1: PR8 + Ovalbumin

$P = 0.001$