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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-OVA1 are protected by injection of 4–8 × 10^6 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: 000–000.

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revious 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 T 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 thought of as universal killers. 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 lpr 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, Tc1 cells are more effective in recruiting host CD4+ T cells 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-21, IL-22, IFN-γ, and TNF, and chemokines, including CCL3, CCL4, CCL5, CXCL9, and CXCL10 (10, 15). We show in this article that these mediators recruit eosinophils, NK cells, macrophages, CD4+ and CD8+ T cells, and B cells to the lung. We show also that the CD8+ effectors could bring about B cell growth and differentiation, activation of innate immunity, and tissue repair, all of which can be assumed to contribute to protection.

Despite, or more likely because of, this enormous range of functions, we were unable to show that any one of the effector mechanisms tested was essential for protection, and we conclude that CD8+ T cells act by multiple redundant protective mechanisms. We do show, however, that the transfer of primed CD8+ T cells rapidly induces a wide range of innate cytokines and chemokines, and we suggest that it is this effect that may be most critical for protection in the early stage of the response.

### Materials and Methods

#### Mouse

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.Ipr, B6 TCR β−/−, 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.Ipr 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-OVA1 (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 × 10^6 EID50 = 4 LD50 and 970 PFU = ~2 LD50 for PR8-OVA1.

#### Viral titers

Mice infected 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

Tc1 and Tc17 effectors were generated from B6.OT-1, B6.OT-1-Thy-1.1, B6.OT-1-1.45.1, B6.OT-1-perforin −/−, B6.OT-1.TRAIL −/−, or clone 4 mice as previously described (15, 16). T cell–depleted APCs (B cell blasts) were prepared by negative selection on MACS columns using FITC-labeled anti-Thy1.2 mAb (53-2.1; eBiosciences) and anti–FITC-MACS beads (Miltenyi Biotech). The B cells were stimulated with LPS (25 μg/ml) and dextran sulfate (25 μg/ml) for 3 d and used as APCs. They were loaded with SINFEKL peptide (10 μg/ml) at 37°C for 30 min, treated with mitomycin C (50 μg/ml) at 37°C for 30 min, and washed 3 times before use. CD8+ T cells from spleens of OT-1 TcR-transgenic mice were enriched by CD8 MACS beads (Miltenyi Biotech) and incubated with SINFEKL, peptide-pulsed B cell blasts (T:B = 1:3) for 4 d. For Tc17 cultures, IL-1β (10 ng/ml; PeproTech), IL-6 (20 ng/ml; PeproTech), porcine TGF-β (3 ng/ml; R&D Systems), IL-21 (80 ng/ml; PeproTech), IL-23 (50 ng/ml; R&D Systems), anti–IFN-γ mAb (11B11, 10 μg/ml), 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/ml MCA, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A. Cells were stained and incubated for 20 min, fixed in 4% paraformaldehyde, and incubated in permeabilizing buffer. Cells were then harvested and incubated with Ab to cell surface markers and then fixed with permeabilization solution (BD Biosciences) for 20 min. Cells were then washed with 1× Perm/Wash buffer (BD Biosciences) and then permeabilized with 1× Perm/Wash buffer with 0.05% Triton X-100 for 10 min. Then cells were stained with Abs to cytokines and clone 4 (BALB.HA) mice were injected into naive B6.Thy1.1 recipients.

In vivo cytotoxicity

In vivo cytotoxicity was assayed as follows: Tc1 and Tc17 effector populations were prepared from OT-1 mice (CD45.2+ Thy1.2+), and 4 × 10^6 cells of Tc17 or Tc1 were injected into naive B6.Thy1.1 recipients. One day after Tc effector injection, recipient mice were injected with 2.5 × 10^7 SINFEKL-pulsed spleen cells stained with 1.25 μM CFSE (CD45.1+ CFSEb), and 2.5 × 10^6 of nonpulsed spleen cells stained with 1.25 μM CFSE (CD45.1+ CFSEa). Twenty-four hours later, spleen cells were harvested and the ratio of surviving CD45.1+ CFSEb to CD45.1+ CFSEa was determined by flow cytometry.

For Fig. 1B, Tc1 and Tc17 effectors were prepared from WT OT-1 (CD45.1+ CD45.2+) and, 4 × 10^6 cells of Tc17 or Tc1 were injected into naive B6.CD45.1 recipients (CD45.1+). One day after Tc effector injection, recipient mice were injected with 2.5 × 10^7 SINFEKL-pulsed WT cells (CD45.2+ CFSEa) and 2.5 × 10^6 of nonpulsed WT cells (CD45.2+ CFSEb). 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 CFSEb targets to CFSEa targets in spleen.

For the ex vivo CTL assay, Tc1 and Tc17 effectors were prepared from OT-1 (CD45.2+), and 4 × 10^6 cells of Tc17 or Tc1 were injected into naive B6.CD90.1 recipients. Two days after Tc effector transfer, recipient mice were infected with 0.2 LD50s of PR8-OVA (1×10^6 diluted). On day 5 of infection, transferred cells were purified from the lung by cell sorting, and 2 × 10^5 cells of infected Tc1 or Tc1 were injected into naive B6.CD45.2 mice. One day after transfer, recipient mice were infected with 2 × 10^8
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Adaptive transfers, lethal infection, weight changes, and survival

B6 mice were injected i.v. with 4, 8, or 16 × 10^6 OT-1 Tc1 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-OV AI 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 percent of initial weight. 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. Twenty-four hours after CFSE-stained cell transfer, the donors and BALB/c as recipients.

Measurement of the host response

B6 OT-1, Thy-1.2 effectors were injected into infected B6.Thy-1.1 mice, which were sacrificed at various time points after influenza infection. Bronchoalveolar lavage was collected by washing the airways five times with 0.5 ml PBS. 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). 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). Lungs were then homogenized in a 100 μl lysis buffer and snap-frozen in liquid nitrogen. Lungs were then homogenized in a 100 μl lysis buffer and snap-frozen in liquid nitrogen.

Depletion of neutrophils, NK-1.1 cells, and Thy-1.1 donor cells

Neutrophils were depleted by i.p. injection on days 2, 5, and 7 of 200 μg/mouse of the monoclonal anti-Gr-1 antibody 103-9c9. NK cells were depleted by injection of 200 μg/mouse anti-NK-1.1 clone PK136 on days 2, 4, and 7. Thy-1.1 donor cells were depleted by a single i.p. injection of 0.1 mg anti-Thy-1.1 (clone 19E12) on days 3, 5, or 8.

DNA and quantitative PCR

RNA was extracted and purified from CD8⁺ T effector cells, using TRIzol (Invitrogen) and RNeasy kit (Qiagen, La Jolla, CA). 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 were designed for the viral polymerase and IFN-γ genes. Cytokine assay

Levels of cytokines and chemokines in lung homogenates (Fig. 8) were determined using mouse multiplex Luminex kits (Invitrogen) 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 then transferred to xylene. Lung tissues were progressively hydrated by transferring them to xylene, alcohol, 96% alcohol, 70% alcohol, and finally water. Ags 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 FeRs 2.4G2 diluted in 0.1% Tween 200, 1% Triton X-100 in PBS. Without washing, primary Abs CD3 (Santa Cruz Biotechnology) clone M-20; proliferating cell nuclear antigen (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 antigen 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 and analyzed by flow cytometry using the same reagents. Tissue sections were mounted with medium for fluorescence with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlington, 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 all play a role in protection. We sought, in this study, to determine the effect of transfer of CD8⁺ effectors on the course of the response in the recipient mice. Our first approach was to determine the correlates of protection mediated by the injected CD8⁺ effector T cells.

Adaptive transfer of CD8⁺ effectors brings about a reduction in 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. 1C) into the lung, and lactate dehydrogenase (Supplemental Fig. 1C) into the lung. Lung function was measured, and respiratory rate (Supplemental Fig. 1D) and respiratory rate (Supplemental Fig. 1E) were measured, and respiratory rate (Supplemental Fig. 1D) and respiratory rate (Supplemental Fig. 1E).

In vivo–generated polyclonal CD8⁺ effectors also provide protection

It is not possible to prepare well-polarized CD8 effector cells in vivo, but polyclonal CD8 effector cells 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 × 10⁶ 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\(_{\gamma}\). 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 day 10, 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 × 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 LD50 PR8-OVA. 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 uninjected 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 × 10^6 Tc17 effectors at day zero and infected with 2 LD50 PR8-OVA 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 for GR-1 (RB6)Ab. A fifth group treated mice were prepared from mice sacrificed at day 8 and analyzed by larger numbers at later times was a correlate of the failure to control infection.

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.
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 TCRβ/TCRδ-deficient recipient mice. Uninjected βδ−/− mice lost weight rapidly, and all died by day 10 when challenged with 3 LD_{50} PR8-OVA_{2} (Fig. 5A). βδ−/− mice injected with either 8 × 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 αβ and γδ T cells and B cells. A total of 8 × 10^6 Tc1 or Tc17 effectors were injected into RAG-2−/− host, which were challenged with 3 LD_{50} PR8-OVA_{2}. 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 βδ−/− 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+ T cells from either WT or perforin-deficient OT-1 mice, and these were injected into WT (Fig. 6A, 6C) or RAG-2−/− (Fig. 6B, 6D) recipients followed for 28 d. Similar results were seen in an earlier experiment in which the mice were challenged with only 0.2 LD_{50}.
FIGURE 6. Perforin-deficient Tc17 protect neutrophil-depleted RAG-2−/− recipients from viral challenge. The design of the experiment was similar in detail to that in Fig. 4B, except that the CD8 effectors were generated with cells from WT or perforin-deficient mice and the recipients were left untreated or were injected as in Fig. 3A to deplete neutrophils. All mice were challenged with 2 LD50 PR8-OVA1. Similar results were seen in two experiments. (A) and (B) indicate the kinetics of weight change for WT mice (A) that were injected with Tc1 WT or perforin-deficient effectors and for RAG-2−/− mice (B) that were injected with Tc1 WT or perforin-deficient effectors. (C) and (D) indicate WT mice that were injected with Tc17 WT or perforin-deficient effectors (C), and for RAG-2−/− mice that were injected with Tc17 WT or perforin-deficient effectors (D). In addition, in (C) and (D), the recipient mice were either neutrophil depleted or left untreated. Similar results were seen in three experiments.

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 survived, suggesting 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 effectors 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 LD50 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-

FIGURE 7. Tc17 donor cells still protect against viral challenge when depleted at day 5 or 8, but not at day 3. A total of 8 × 10^6 in vitro–generated Tc1 (A) or Tc17 (B). OT-1 effectors were injected into groups of five naive WT B6 recipients and challenged 1 d later with ∼1 LD50 PR8-OVA. Donor T cells were from OT-1.Thy-1.1 mice and were depleted by the injection of anti-Thy-1.1 at day 3, 5, or 8. Weight changes and survival were followed for 15 d. Data displayed in (A) and (B) all come from the same experiment, but for ease of display, the weight change curves are separated into two parts. The weight changes for untreated mice (no transfer) and the isotype control for the CD8 effector treated mice are shown in the left panels of (A) and (B). The effects of depletion at 3, 5, and 8 d are shown in the middle panels. The corresponding survival curves for (A) and (B) are shown in the right panels. (C) Representative example of the efficacy of donor cell depletion using anti–Thy-1.1 injection. Similar results were seen in two experiments.

FIGURE 8. Tc17 donor cells provide bystander protection against viral challenge. A total of 8 × 10^6 in vitro–generated Tc1 (A) or Tc17 OT-1 (B) effectors were each injected into three groups of mice. One was challenged PR8, a second with PR8-OVA, and the third with both viruses (n = 4). (C) The third group of mice was injected with Tc1 effectors and 100 μg LPS-free OVA i.n. instead of PR8-OVA (n = 5). Weight changes and survival were followed for 15–30 d.
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.

numbers of CD8+ T cells were seen at the peak of the response of a normal mouse to influenza infection. The dominant subset is the IFN-secreting Tc1-like, which reaches numbers 50-fold greater than those of the Tc17-like subset (16). There were \( \sim 10^6 \) for IFN-γ-secreting Tc1-like cells, but only \( 8 \times 10^5 \) for IL-17-secreting Tc17-like cells. Nonetheless, the numbers of Tc17 cells expand almost 100-fold during the response, and we conclude they must also play some significant role in these responses (16).

The goal of this study was to define the roles of these cells by determining how the adoptively transferred CD8+ effector T cells protected recipient mice against otherwise lethal influenza infection in recipient mice. In the model used, we transferred large numbers of CD8+ effectors, in vitro, from SIINFEKL/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). Effector cells are present 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.
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 intradermal 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 injected, but at the dose selected of 8 × 10\(^9\) Tc17 effector cells per mouse, there was no loss of protection against a viral dose that killed four of five of the unprotected WT recipients. We also recognized the caveat that donor T cells injected into RAG-2–/– mice might expand much more extensively than in WT recipients, and the larger numbers of effector cells were perhaps able to make up for other deficiencies and may also rely on a different mix of protective mechanisms from WT effectors in WT hosts, thus invalidating any simple interpretation of the result.

The deletion of dendritic cells using diphtheria toxin and CD11cDTR recipient mice led to no loss of protection, but the collateral effects of the treatment were excessive inflammation and precluded the drawing of any firm conclusion (data not shown). The deletion of macrophages using clodronate liposomes led to the death of all of the mice (data not shown), even those challenged with a sublethal dose of virus, and it was therefore not possible to conclude whether removal of the macrophages had any effect on the protection provided by injection of CD8 effectors. We previously showed that Tc17 secrete IL-22 (16, and data not shown) and obtained some evidence that Tc17 effectors accelerated type II epithelial cell proliferation (data not shown), but Ab to IL-22 was without effect on protection (data not shown), which suggested IL-22–mediated repair of lung epithelium was not essential.

We were thus not able to identify any essential single or group of mechanisms necessary for protection. Therefore, it would appear that protection is afforded by multiple pathways, many of which are redundant, and that there is no single key mechanism that is essential for survival. Our major conclusion is that CD8 carries out a very large number of effector functions providing multiple layers of redundant protection against otherwise very dangerous pathogens.

Some further insight was gained in experiments that showed that there was an Ag-specific phase in the protective mechanism that was followed by an Ag-independent phase starting around day 4. Depletion of donor cells at later time points did not interfere with protection, and the addition of a small amount of the PR8-OVA\(_3\) virus to PR8 was sufficient for the SIINFEKL–specific OT-1 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 x 10^6 in vitro generated Tc1 or Tc17 OT-1 effectors or left uninjected and challenged one day later with approximately 0.2LD50 PR8-OVA1. 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 in vitro. Tc1, Tc2 and Tc17 effectors were prepared and RNA message levels for FasL and TRAIL (product of tnfsf10) 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 x 10^6 in vitro generated Tc17 OT-1 effectors were injected into groups of 5 naïve B6 recipients which were challenged with 2LD50 PR8-OVA1. 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, \(8 \times 10^6\) 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 \(1\text{LD}_{50}\) A/PR8 a second with \(1\text{LD}_{50}\) PR8-OVA\(_1\) 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 \(\mu\text{g}\) LPS free ova instead of A/PR8-OVA\(_1\) (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 4

![Graph showing PA copies per lung (Log₁₀) over days 3 and 7 for different groups: Tc1: PR8-OVA₁, Tc1: PR8, and Tc1: PR8 + Ovalbumin. The graph indicates a statistically significant difference (P = 0.001) between the groups.]