CD4 T Cell-Mediated Protection from Lethal Influenza: Perforin and Antibody-Mediated Mechanisms Give a One-Two Punch

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Influenza infection poses a serious health threat, particularly since new avian influenza viruses may develop which can be directly transmitted to humans causing significant mortality. In addition, influenza infection is responsible for 30–50 thousand deaths in the elderly, neonatal, or immunocompromised populations in the United States per year (1). At this time, there is widespread concern that a pandemic may arise in which the hemagglutinin (HA) or neuraminidase (NA) outer proteins from H5N1 or other avian influenza viruses either mutate or combine with the current circulating human influenza strains and confer greater transmission. Current vaccines generate neutralizing Abs that target the outer HA and NA proteins of influenza viruses now in circulation and are highly strain specific. However, the emergence of a reassortant virus, with different outer proteins, would render the current vaccines ineffective, resulting in the majority of the population lacking immunity to the new strain (2). A promising additional approach would be to develop vaccines that promote cell-mediated immunity and target internal proteins of influenza, such as nucleoprotein, that are more conserved among different influenza A viruses (3). However, some argue that cell-mediated, or heterosubtypic, immunity will not be as vigorous as humoral immunity, and thus, less efficacious against a highly pathogenic or pandemic strain of influenza (4). Therefore, it is of pressing importance to evaluate the potential of T cell immunity to protect from the deadly consequences of a virulent influenza strain.

Following a primary infection, both CD8 and CD4 T cells are thought to contribute to protection against reinfection with heterosubtypic influenza A viruses that share internal proteins, but differ in their HA and NA subtypes (5). In mouse models of primary infection, less pathogenic isolates of influenza A can be cleared in the absence of CD8 T cells, CD4 T cells, or B cells (reviewed in Ref. 6). However, infection with more pathogenic strains of influenza require both CD4 and CD8 T cells, or B and T cells together for complete clearance (6, 7). Therefore, resolution of highly pathogenic influenza viral infection is a complex process that may require the synergy of cell-mediated and humoral immune responses.

CD4 T cells are important in regulating immune responses to infection, and these cells can be further subdivided based on the cytokine profiles that they produce (8–10). When IL-4 is present early in the immune response, naïve CD4 cells differentiate to produce IL-4, IL-5, and IL-13. These Th2 cells can provide B cell help and preferentially drive IgG1 and IgE Ab production. In contrast, the presence of IL-12 induces naïve CD4 cells to secrete IFN-γ and differentiate into Th1 cells that can mediate cellular immune responses such as delayed-type hypersensitivity reactions. Influenza infection generally produces a cytokine milieu that favors the generation of a Th1 response in which effector CD4 cells produce IFN-γ and TNF-α (11, 12). Despite Th1 polarization, the response to influenza is also characterized by the production of a robust Ab response of all IgG subtypes as well as IgA (13).

Early attempts to dissect CD4 effector mechanisms that combat influenza used Th1- or Th2-polarized CD4 T cell clones. Most CD4 cell clones that were isolated after influenza infection demonstrated a Th1 phenotype (14, 15), but Th2 clones could also be generated after immunization with inactivated virus in adjuvant...
These influenza-specific cloned populations differed in their effector profiles and functional capacity; where Th1 clones were shown to be more effective than Th2 clones in protection against a lethal challenge with A/Japan/57 virus (14, 16). However, this approach had several disadvantages, and the precise mechanisms whereby these T cell clones promoted survival were not elucidated. One drawback of these studies is that cloned T cells that have been repeatedly stimulated in vitro with Ag and cytokines may not reflect the phenotypic characteristics of an effector cell that has undergone only a primary stimulation. In addition, cloned T cell lines could not be easily manipulated, and with the advent of TCR transgenic (Tg) mice and gene targeting, primary CD4 T cells can now be generated that lack important effector molecules to specifically study mechanisms.

To investigate the functional potential of CD4 T cells in response to lethal infection, we isolated naive CD4 T cells from TCR Tg mice that were specific for a HA peptide from influenza A/Puerto Rico/8/34 (PR8). In the studies described here, naive CD4 cells were stimulated in vitro for 4 days with HA peptide-pulsed APC and Th1-polarizing cytokines, resulting in the generation of a well-characterized, homogeneous population of influenza-specific primary CD4 effectors (17). These CD4 effectors can be derived from wild-type (WT) or gene-targeted donors and are adoptively transferred into immunocompromised hosts to better identify important effector molecules and mechanisms used by primed CD4 cells to mediate protection to an otherwise lethal influenza infection.

We demonstrate in this study that CD4 effectors use multiple mechanisms to promote protection against lethal influenza infection. CD4 effectors confer protection against a highly pathogenic influenza virus in an IFN-γ-independent manner that does not require host T cells. However, CD4-mediated protection does require B cells and the ability of CD4 effectors to accelerate and enhance the production of anti-influenza Abs. Moreover, in B cell-deficient mice, passive immune serotherapy can replace B cells to specifically study mechanisms.

Materials and Methods

Mice

TCR Tg mice in which CD4 cells recognize a peptide 126-138 of HA protein from PR8 influenza were a gift from Dr. D. Lo (The Scripps Research Institute). BALB/c By and BALB/c mice on the BALB/c background have been described previously (19) and were bred to TCR Tg mice at the Trudeau Institute Animal Breeding Facility. Pfn-/- mice were a gift from Dr. J. Harty (University of Iowa, Iowa City, IA) and were bred to BALB/c mice on the BALB/c background. All experimental animal procedures were conducted in accordance with the Trudeau Institute Animal Care and Use Committee guidelines.

Medium and peptides

All cells were grown in RPMI 1640 (Invitrogen Life Technologies) containing 2 mM l-glutamine, 100 IU penicillin, 100 μg/ml streptomycin (all obtained from Invitrogen Life Technologies) 10 mM HEPES (Research Organics), 50 μM 2-ME (Sigma-Aldrich), and 8% FBS (HyClone). HA peptide 126–138 (HNTNGVTAACSHE), HA peptide 118–128 (H9253), HA peptide 118–128 (H11003), and OVA peptide 323–339 (I9QVHAAHAEINAGR) were synthesized by New England Peptide.

Isolation of CD4 T cells and in vitro activation

Naive CD4 cells were isolated from TCR Tg mice using a positive selection protocol with anti-CD4 beads (clone GK1.5) according to the manufacturer’s protocol (Miltenyi Biotec). B cell blasts were generated as previously described (21), pulsed with 5 μM HA 126–138 peptide and combined with naive CD4 cells at a 1:1 ratio to give a final concentration of 3 × 106 cells/ml. IL-2 at 11 ng/ml, IL-12 at 2 ng/ml, and anti-IL-4 (clone 11B11) at 10 μg/ml were added to polarize CD4 cells to the Th1 phenotype characteristic of high IFN-γ secretion (17). Additional IFN-γ at 100 ng/ml was added to IFN-γ-/- cultures to inhibit IL-4 and IL-5 secretion (Ref. 22 and see Fig. 3A). After 2 days, an equal volume of complete medium containing IL-2 at 11 ng/ml was added, and cultures were incubated for an additional 2 days. To assay for activation and polarization, WT, IFN-γ-/- or Pfn-/- CD4 effectors at 1 × 106 cells/ml were restimulated with immobilized anti-CD3 for 48 h, and supernatants were analyzed for cytokine secretion using Beadlyte cytokine beadarrays (Upstate Signaling) according to the manufacturer’s instructions. Cytokines were detected using a Luminex 100 (Luminex). Standard curves were analyzed and unknowns were calculated using Prism software (GraphPad).

CD4 effector transfer and infection of mice

A total of 5 × 106 CD4 effectors in PBS was injected via tail vein into BALB/c mice, unless otherwise noted. Eighteen to 24 h after CD4 effector injection, mice were infected intranasally with 5000 egg infectious units (EIU) PR8 influenza virus in 50 μl of PBS, unless otherwise indicated. Mice were monitored for weight loss and survival over the course of the experiment. Mice were euthanized when they became moribund according to the Trudeau Institute Animal Care and Use Committee guidelines.

FACS analysis of donor and host populations

Lethally infected BALB/c. Thy1.1 mice were sacrificed at various times postinfection. Lungs were perfused with PBS, dissociated, and single-cell suspensions were incubated with fluorescently labeled anti-CD4 (clone RM4-4) and anti-Thy-1.2 for 30 min in FACS buffer (PBS with 1% BSA, 0.1% Na azide) at 4°C to mark donor cells. Lung cells were also incubated with FITC-labeled anti-CD8α (clone 53-6.7) to identify host CD8 cell populations. Cells were fixed in 1% paraformaldehyde and analyzed using a BD Biosciences FACSCalibur, and data were processed using FlowJo software (Tree Star).

Quantitation of viral RNA by TaqMan PCR

Viral RNA was detected in a manner similar to previously published protocols (23, 24). RNA was isolated from lung homogenates using TRIzol (Sigma-Aldrich) and 2.5 μg of RNA was reverse-transcribed into cDNA using random hexamers and Superscript II reverse transcriptase (Invitrogen Life Technologies). Fifty nanograms of cDNA was then used for amplification by quantitative real-time PCR (ABI Prism 7700; Applied Biosystems). The following primers and probe were used: 5'-CGGTCTCAAAATCTCCTTGCTGAT-3' (forward), 5'-CAATGTTGTCCTTCCATTCCA-3' (reverse), and 5'-6-FAM-CCAAGCTAGAAAGAGGGAATACCGT-3' (probe). The acid polymerase (PA)-gene copy number was calculated based on the total amount of RNA in each lung sample.

ELISA for detection of anti-influenza Abs

ELISA plates were coated overnight at 4°C with live influenza PR8 virus diluted 1/200 (5 × 106 EIU/ml) in PBS. Plates were washed four times with PBS and 200 μl/well of PBS containing 2% FBS, and 10 mM HEPES was added to block nonspecific binding sites. Serum was then added in blocking buffer starting at a 1/20 dilution and serially diluted 2-fold. After 2–3 h at 20°C, alkaline phosphatase-conjugated goat anti-mouse IgG
of unpulsed in naive)

unpulsed in transferred)/(percentage of peptide pulsed in naive/percentage
of [3H]thymidine was measured using a beta plate scintillation counter (Wal-
dressed to effectors at a concentration of 1 1002 peptide, or irrelevant peptide as a control, was added at a final concentra-
tion of 2 µg/ml. After 4 h at 37°C, plates were harvested, and incorporated
[3H]thymidine was measured using a beta plate scintillation counter (Walc.
In some experiments, concanamycin A (CMA) (Sigma-Aldrich) was
added to inhibit Pfn exocytosis (25). In other experiments, anti-Fas ligand
(FasL) at 10 µg/ml was added to inhibit Fas-mediated killing (26).
Percentage of specific cytotoxicity was calculated as (spontaneous cpm −
experiment cpm)/spontaneous cpm × 100.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed as described with minor modi-
fications (27). Three days posteffector transfer, naive BALB/c splenocytes
were pulsed with the class II-restricted HA126–138 peptide for 1 h at 37°C
and labeled with 5 µM CFSE (Molecular Probes). Naive BALB/c spleno-
cytes were also pulsed with the class I-restricted HA518–528 peptide for 1 h
at 37°C and labeled with 0.5 µM CFSE. Targets were combined at a 1:1 ratio,
and a total of 5 × 106 cells/mouse were injected i.v. Eighteen hours
after target injection, mice were sacrificed, spleens were removed, and red
cells were lysed and resuspended in FACS buffer. Cells were analyzed with
a BD Biosciences FACSCalibur, and data were processed using FlowJo
software (Tree Star). Percentage of specific cytotoxicity was calculated as
follows: 100 − (((percentage of peptide pulsed in transferred/percentage
of unpulsed in transferred)/(percentage of peptide pulsed in naive/percentage
of unpulsed in naive)) × 100).

Statistical analyses

Statistical analyses were performed by Prism 4.0 software (GraphPad) us-
ing Student’s t tests for parametric data. Survival curves were plotted and
analyzed by Prism 4.0 software using the Kaplan-Meier method and
log-rank test.

Results

Primed CD4 effectors protect against lethal influenza infection
in an Ag-specific manner

Previous studies have demonstrated that repeatedly stimulated
CD4 T cell clones transferred to intact mice could protect against
a lethal dose of influenza A/Japan/57/14. We first investigated
whether CD4 effectors primed in vitro could promote survival
against a highly pathogenic influenza strain such as PR8. In these
experiments, we use the term “protection” to signify survival for
>20 days after infection with a dose of influenza that routinely
kills 80% or more of normal, unprimed mice. Naive CD4 cells
were isolated from TCR Tg mice, and effectors were generated by
priming with influenza HA peptide-pulsed B cell blasts and Th1-
polarizing cytokines for 4 days. CD4 effectors stimulated in this
way show decreased levels of CD62L and increased expression of
CD43, CD11a, and CD49d compared with naive cells (data not
shown). The cell surface profile of in vitro-primed CD4 effectors
was similar to that seen when CD4 cells are primed in vivo during
a sublethal dose of influenza (12). A total of 5 × 106 in vitro-
primed CD4 effectors was transferred into adoptive hosts that were
subsequently infected with 5000 EIU (~2 LD50) PR8. Fig. 1A
demonstrates that Th1-polarized, HA peptide-specific CD4 cells
protected BALB/c mice against an otherwise lethal dose of influ-
enza PR8. In contrast, OVA-specific Th1 CD4 effectors generated
from DO11.10 TCR Tg mice did not provide protection. Thus,
CD4 T cell-mediated protection is Ag specific and is not due to
nonspecific activation of the transferred cells at the site of infec-
tion. Equivalent numbers of naïve HA-specific cells did not pro-
mote survival after a similar challenge with PR8 (Fig. 1B), dem-
onstrating that CD4 cells must first be primed to provide protection.
Furthermore, the protective effect (as measured by the
fraction of surviving mice) of primed CD4 cells was shown to be
dependent on the number of cells transferred (Fig. 1C). We found
that transfer of 5 × 106 cells provided optimal protection, whereas
a 10-fold lower number of CD4 effectors was not effective. There-
fore, primed but not naive, CD4 T cells effectively promote sur-
vival to lethal influenza infection in an Ag-specific manner.

FIGURE 1. Primed CD4 effectors protect against lethal influenza infec-
tion in an Ag-specific manner. A total of 5 × 106 HA-specific (●) or
OVA-specific (△) cells was transferred to normal BALB/c mice (n =
5/group) and 18 h later, infected with 5000 EIU (2 LD50) PR8 virus (A). B.
A total of 5 × 106 naive HA-specific cells (●) or no cells (△) was injected
into BALB/c mice (n = 5/group) followed by infection with 5000 EIU PR8
virus. C. A total of 5 × 106 (●), 1 × 106 (□), or 5 × 105 (◆) CD4 effector
cells was injected into BALB/c mice (n = 5/group) that were then infected
with 5000 EIU PR8. Control mice did not receive cells (△). Mice were
monitored for survival and were euthanized when they became moribund
in accordance with Trudeau Institute’s Animal Care and Use Committee
guidelines.
CD4 effectors localize to the lung, decrease viral titer, and provide protection independent of host T cells

Visualization of the anti-influenza CD4 response in situ suggests that naive cells are primed in the draining lymph nodes (LN), and then highly differentiated effectors migrate to the lung and release cytokines in response to antigenic challenge (12). Earlier studies in our laboratory suggest that in vitro-generated CD4 effectors transferred to unprimed mice enter both secondary lymphoid and nonlymphoid sites within 24 h, even in the absence of inflammatory stimuli (28). Because CD4-mediated protection occurred only when the cells were primed (Fig. 1), it is likely that CD4 effectors migrate to the lung early in the response and act directly to promote recovery. To determine the localization and kinetics of CD4 effector migration to nonlymphoid sites, Thy1 disparate CD4 effectors were transferred into BALB/c mice that were then infected with PR8 virus. The absolute number of donor CD4 effectors in the lung was determined by FACS analysis at various times postinfection and is shown in Fig. 2A. CD4 effectors were recovered from the lung as early as 1 day after infection, and the absolute numbers of CD4 effectors peaked at day 2 postinfection (Fig. 2A). By day 4 postinfection, the number of CD4 effectors had decreased ~4-fold from the day 2 peak, and few donor T cells were detectable 1 wk after infection. This pattern was also observed in the draining LN, but with ~4-fold lower donor cell numbers than in the lung (data not shown). This result suggests that primed CD4 T cells exert their protective effects within the first 4 days after infection.

The rapid influx of CD4 effectors to the site of infection suggested that these cells might function early to reduce viral titers. It has been demonstrated that cytolytic CD8 T cells attack virally infected epithelium and are primarily responsible for decreasing viral titers (29). However, it has also been shown that CD4 cells can promote viral clearance in the absence of CD8 cells (30) and that memory CD4 cells residing in the respiratory tract also act to decrease viral titer (31). To determine whether transfer of CD4 effectors had an impact on viral replication in the lung, a quantitative PCR-based assay that detects the influenza A PA gene copy number was used (23). Fig. 2B shows PA gene copy number per lung at day 4 postinfection and demonstrates that mice given CD4 effectors had significantly \( p < 0.05 \) less viral RNA than PBS-treated controls, suggesting that CD4 effectors did act in some way to reduce viral titers.

Results shown thus far indicated that CD4 effectors localized to the lung and decreased viral titers; however, it was not clear whether the transferred cells functioned directly on virally infected cells, or indirectly via actions on host T cells. To determine whether CD4 effectors accelerated the migration of host T cells into the lung, or increased the magnitude of the host response after lethal infection, host CD8 and CD4 cell populations were enumerated using FACS analysis. Fig. 2, C and D, show that the number of host CD8 or CD4 cells do not increase substantially until 7 days postinfection, regardless of the presence of CD4 effectors. Although addition of CD4 effectors had no impact on the number of host T cells in the lung, it was still unclear whether the host T cell response was contributing to the decrease in viral burden. To determine whether host T cells were required for CD4-mediated protection, CD4 effectors were transferred to BALB/c athymic nu/nu recipients, lacking both CD4 and CD8 cells, followed by lethal PR8 infection. Fig. 2E demonstrates that T cell-deficient mice given CD4 effectors survived a 5000 EIU dose of PR8, whereas T cell-deficient mice given PBS succumbed to lethal influenza infection. Taken together, these data demonstrate that protection mediated by CD4 effectors did not require host T cells, suggesting that CD4 effectors may be acting in a direct mechanism to decrease viral titers at the site of infection.

Protection mediated by CD4 effectors does not require IFN-γ in the priming or effector phase

Using an adoptive transfer model, our laboratory has previously demonstrated that naive TCR Tg CD4 T cells specific for HA of PR8 influenza respond vigorously to sublethal (500 EIU) infection (12). These studies demonstrated that naive CD4 cells proliferate and differentiate in the draining LN and spleen and acquire a Th1 effector profile. The most differentiated cohort of effectors migrate to the lung and produce IFN-γ, but little to no IL-2 at the peak of the response (12). Based on these data, we hypothesized that CD4-derived IFN-γ might be important for CD4-mediated protection to
lethal influenza infection. To test this theory, we generated effectors from TCR Tg mice that were deficient in IFN-γ. Because WT CD4 effectors were normally exposed to IFN-γ during effector generation in vitro, we generated IFN-γ−/− CD4 effectors that were also primed in the presence of exogenous IFN-γ during the 4-day culture period and compared these cells to IFN-γ−/− effectors primed in the absence of exogenous IFN-γ. FACS analysis of the cell surface markers CD62L, CD25, CD43, and CD27 did not reveal differences in the activation state between WT and IFN-γ−/− effectors generated with or without IFN-γ (data not shown). As shown in Fig. 3A, both WT and IFN-γ−/− CD4 effectors produced similar levels of TNF-α, RANTES, and IL-10. However, IFN-γ−/− effectors generated under Th1-polarizing conditions, but without exogenous IFN-γ during the priming phase, produced some IL-4, IL-5, and IL-6 (∼1 ng/ml) as has been described previously (22). IFN-γ−/− cells exposed to IFN-γ during the culture period lost the ability to produce IL-4 but still secreted IL-5, albeit at lower levels. Interestingly, IFN-γ−/− effectors primed in the presence or absence of IFN-γ produced more of the inflammatory cytokine IL-17 than WT cells.

To determine whether CD4-derived IFN-γ was required for protection to lethal influenza infection, WT and IFN-γ−/− effectors grown in the absence or presence of IFN-γ were transferred to BALB/c mice that were then infected with a lethal dose of influenza. Fig. 3B shows weight loss (top panels) and survival (bottom panels) of mice infected with 5,000 or 10,000 EIU (∼5 LD50) PR8 over a 28-day period. All mice lost weight at the same rate in the first 6–7 days postinfection; however, mice that received CD4 effectors began to recover at ∼7-day postinfection, whereas untreated mice continued to lose weight and eventually succumbed at 5,000 EIU PR8. Increasing the viral dose to 10,000 EIU induced a greater weight loss in all mice, but again, mice that received CD4 effectors began to recover at approximately day 10 postinfection. Importantly, there was no difference in the ability of WT or IFN-γ−/− effectors to alter weight loss or promote survival, and furthermore, IFN-γ−/− effectors primed with or without IFN-γ could protect mice against lethal infection at both doses of PR8.

Because it was possible that IFN-γ could be more important for protecting mice against higher doses of pathogenic virus, mice were given CD4 effectors and subsequently infected with 20,000 EIU PR8. At this high dose (∼10 LD50), all mice lost ∼35% of body weight, and mice given CD4 effectors did not begin to recover until day 15 postinfection. Both WT and IFN-γ−/− effectors were equally effective in abrogating weight loss and promoting survival at this high dose, and no difference in the protective capacity of the effectors was observed (Fig. 3C). Together, these data demonstrate that protection mediated by CD4 effectors does not require IFN-γ in the priming or effector phase, suggesting that CD4 cells use other mechanisms to promote survival against lethal challenge.

**CD4 effectors induce protective anti-influenza Abs**

A hallmark of CD4 effector function is their ability to promote B cell differentiation and Ab production. Therefore, we hypothesized that increased Ab production, driven by CD4 effectors, may be important for CD4-mediated protection. To evaluate the impact of CD4 effector transfer on Ab production by host B cells, we assayed influenza-specific Ab in serum from lethally infected mice given CD4 effectors or PBS as a control. In BALB/c mice that did not receive effectors, anti-influenza titers were negligible at day 6 postinfection, and total anti-influenza IgG titers were <100 by the time mice became moribund. Transfer of CD4 effectors significantly increased anti-influenza IgG Ab titers in response to lethal PR8 infection compared with mice given PBS (Fig. 4A). Indeed, anti-influenza IgG titers in lethally infected mice given CD4 effectors were 10- to 50-fold higher (p < 0.01) than in mice given PBS and subsequent lethal infection. Cytokine production by CD4 cells is known to influence the Ab isotype profile to T-dependent Ags, and IFN-γ can enhance class switching to IgG2a (32). Consistent with this, Fig. 4B demonstrates that mice given WT CD4 effectors produce significantly higher amounts of anti-influenza IgG2a (p = 0.01) and less IgG1 compared with mice given IFN-γ−/− effectors. Although the ratio of anti-influenza IgG2a to IgG1 was different in mice receiving WT or IFN-γ−/− effectors, the ability of CD4 effectors to enhance early Ab production by B cells was not dependent on IFN-γ.

If CD4-dependent production of anti-influenza Ab is required for CD4-mediated protection, then B cell-deficient mice should not survive a lethal challenge with PR8. Fig. 4C shows weight loss and survival curves of B cell-deficient (JHD) mice that received PBS, WT, or IFN-γ−/− effectors before lethal infection. Fig. 4C demonstrates that WT CD4 effectors were unable to protect JHD mice from lethal infection. Although CD4 effectors did not provide complete protection in B cell-deficient mice, JHD mice receiving IFN-γ−/− CD4 effectors were able to significantly (p = 0.005) delay mean time to death compared with JHD mice given PBS alone.

The observation that CD4 effectors did not protect B cell-deficient mice from lethal infection suggested that either B cells, or the Abs they produce, were required for CD4-mediated protection to

**FIGURE 3.** Protection mediated by CD4 effectors does not require IFN-γ in the priming or effector phase. A, Cytokine release, after 48-h restimulation with immobilized anti-CD3, by WT effectors (•), IFN-γ−/− effectors cultured in the presence (■) or absence (dotted bar) of exogenous IFN-γ after the initial 4-day culture. B, A total of 5 × 10^6 WT cells (●), IFN-γ−/− cells cultured in the presence (▲), or IFN-γ−/− cells in the absence (○) of exogenous IFN-γ was injected i.v. into BALB/c mice (n = 5/group) that were then infected with 5,000 (2 LD50) or 10,000 (5 LD50) EIU PR8 18 h later. C, A total of 5 × 10^6 WT cells (●) or IFN-γ−/− cells cultured in the presence of exogenous IFN-γ (●) were injected into BALB/c mice and infected with 20,000 EIU (10 LD50) PR8 18 h later. Control mice did not receive CD4 effectors (Δ). Mice were monitored for weight loss and morbidity over time.
CD4 effectors induce protective anti-influenza Abs. BALB/c mice were given 5 × 10^6 WT (■) or IFN-γ^-/- (○) effectors, or PBS (●) as a control. Mice were then infected with 5000 EIU (2 LD₅₀) PR8 virus, and serum was collected at 6, 8, 11, 13, and 20 days postinfection. Shown in A is the mean end point anti-influenza IgG titer of three individual mice (±SD), and B shows the mean end point anti-influenza IgG1, IgG2a, and IgG2b titer of four mice ± SD at day 20 postinfection. This experiment was repeated twice with similar results. A total of 5 × 10^6 WT (■) or γ^-/- (○) effectors were injected into BALB/c JHD mice (n = 5/group), and 18 h later, mice were infected with 2500 EIU PR8. Control mice received PBS (●). C shows weight loss and survival of JHD mice receiving CD4 effectors only. PBS control and mice infected with γ^-/- CD4 effectors demonstrated a statistically significant difference in survival (p < 0.005) by the Kaplan-Meier log-rank test. This experiment was repeated twice with similar results and statistical differences. JHD mice receiving CD4 effectors were given naïve serum (D), 10 μl of immune serum/mouse (E), or 20 μl of immune sera/mouse (F) 7 days postinfection. Mice receiving IFN-γ^-/- effectors and 10 μl of immune serum showed a statistically higher survival rate than mice receiving 10 μl of immune serum alone (p < 0.05). These experiments were repeated with a different batch of immune sera with similar results.

CD4 effectors are cytolytic in vitro and in vivo and contribute to Ab-independent protection

The above evidence suggested that CD4-mediated protection did not require IFN-γ or host T cells. However, the plateau in weight loss consistently observed at day 5–10 postinfection, combined with a significant delay in death (Fig. 4C), suggested that CD4 effectors exerted some level of protection in the early stages of infection. There is increasing evidence that CD4 cells can acquire cytolytic activity during infections in mice (33) and humans (34). If CD4 effectors developed cytolytic activity during the 4-day in vitro-priming protocol, they may be able to directly kill virally infected cells. To determine whether primed CD4 effectors acquired cytolytic activity, we examined the ability of in vitro-generated CD4 effectors to kill HA peptide-coated targets in a 4-h in vitro assay (35). Fig. 5A (left panel) demonstrates that both WT and IFN-γ^-/- CD4 effectors were highly cytolytic to peptide-pulsed A2O target cells. This lytic activity was Ag specific because unpulsed A2O cells were not killed. Furthermore, IFN-γ^-/- CD4 effectors had a greater capacity to kill peptide-coated targets compared with WT CD4 effectors (Fig. 5A) and were as potent as CD8 effectors in killing target cells (data not shown).

Peptide-specific cytolytic activity can occur via two main mechanisms that result in DNA fragmentation and apoptosis of the target cell (36). One mechanism involves the degranulation of intracellular vacuoles and secretion of Fas and granzymes, whereas the other involves receptor-mediated binding of FasL on the effector cell to Fas on the target cell (37). These two mechanisms of cellular lysis can be distinguished in vitro by the action of the drug CMA, which blocks exocytosis of intracellular vacuoles, thus inhibiting the Fas-dependent pathway (25), or by addition of a blocking Ab to FasL (38). Because Fas:FasL is known to be a major mechanism of lysis used by CD4 cells in vitro (39), anti-FasL (38) was added during the cytotoxicity assay to block Fas:FasL-mediated killing. Fig. 5A, right panel, shows that blocking of Fas:FasL interactions using anti-FasL Ab did not inhibit CD4-mediated cytotoxicity of peptide-coated A2O cells, even in the 18-h assay, indicating that Fas:FasL was not a major mechanism of cytolytic activity used by these cells.

To determine whether HA-specific CD4 effectors generated in vitro could kill targets using a Pfn-dependent mechanism, we used CD8 cells from HA-specific TCR Tg mice, also primed in vitro under type 1 conditions, as a positive control for Pfn-mediated cytolytic activity.
CD4-MEDIATED PROTECTION TO LETHAL INFLUENZA

It was important to confirm that CD4 effectors retained cytolytic activity upon adoptive transfer in vivo. Therefore, we coinjected CFSE$^{bsh}$ targets pulsed with HA$_{126-138}$ peptide (recognized by CD4 effectors) and CFSE$^{low}$ targets pulsed with HA$_{518-528}$ peptide (recognized by CD8 effectors) to evaluate lytic activity in vivo (27). In this assay, the disappearance of peptide-pulsed CFSE-labeled targets in mice given effectors compared with naive mice indicates peptide-specific cytotoxicity. Fig. 5B shows that the HA$_{126-138}$ peptide-loaded targets were preferentially deleted in mice given CD4 effectors (compare 0.43 to 0.97%), whereas the HA$_{518-528}$ peptide-pulsed targets disappeared in mice given CD8 effectors (compare 0.19 to 1.04%). IFN-$\gamma^{-/-}$ CD4 effectors also lysed HA$_{126-138}$ peptide-pulsed targets (compare 0.55 to 0.97%) upon adoptive transfer. Percentages of the resulting CFSE$^{bsh}$ and CFSE$^{low}$ targets were converted into percentage of cytotoxicity, as described in Materials and Methods, and are shown as a bar graph in Fig. 5B. This experiment was repeated using an irrelevant class II binding peptide (OVA$_{323-339}$) as a negative control with similar results (data not shown). Therefore, deletion of HA-specific, class II peptide-pulsed targets indicated that CD4 effectors retain peptide-specific cytolytic activity in vivo.

The ability of CD4 effectors to acquire Pfn-mediated cytolytic activity led us to postulate that these cells may promote survival independent of Ab production by B cells. Because B cell-deficient mice succumb to lower doses of influenza PR8 (41), we evaluated CD4-mediated protection after JHD mice were infected with 1500 EIU PR8 virus, a 2-fold lower dose than that used in the previous experiments (Fig. 4). Fig. 5C demonstrates that 1500 EIU of PR8 virus induced a rapid, progressive weight loss and was fatal to all untreated B cell-deficient mice by day 15 postinfection. In contrast, JHD mice that received CD4 effectors again showed a plateau in weight loss from days 5–10 but continued to regain weight, and, ultimately, 80% survived an otherwise lethal dose of virus. Therefore, CD4 effectors alone provided protection, without the addition of passive sera, suggesting that cytolytic activity of CD4 effectors may contribute to Ab-independent mechanisms of protection to lethal influenza.

CD4 effectors demonstrate Pfn-mediated cytolytic activity that is required for Ab-independent protection to lethal infection

To directly test the requirement for Pfn in CD4-mediated protection, CD4 effectors were generated from TCR Tg mice deficient in Pfn (Pfn$^{-/-}$) (42). Pfn$^{-/-}$ effectors displayed an activated phenotype (data not shown) and secreted high levels of IFN-$\gamma$, IL-10, TNF-$\alpha$, and RANTES, similar to WT effectors (Fig. 6A). However, Pfn$^{-/-}$ effectors did not lyse peptide-pulsed A20 target cells in a 4-h cytotoxicity assay, whereas WT effectors demonstrated high, Ag-specific cytolytic activity (Fig. 6B, left panel). This result confirmed the data shown in Fig. 5A and verified the ability of CD4 effectors to lyse peptide-specific targets via Pfn-mediated cytotoxicity. Interestingly, Pfn$^{-/-}$ effectors acquired FasL-mediated cytolytic activity in an 18 h assay, which was blocked by an Ab to FasL (Fig. 6B, right panel). Again, lytic activity by WT effectors was not blocked by anti-FasL, indicating that the major mechanism of target cell lysis used by CD4 cells involves Pfn.

Because CD4 effectors demonstrated Pfn-mediated lysis in vitro, and exhibited lytic activity in vivo, we tested whether Pfn$^{-/-}$ CD4 effectors could mediate protection to lethal influenza. Intact BALB/c mice were injected with WT or Pfn$^{-/-}$ effectors and subsequently infected with very high doses of PR8 virus. Fig. 6C demonstrates that Pfn$^{-/-}$ effectors were less effective than WT

cytolytic function (40). CMA was added during the 4-h cytotoxicity assay to specifically block Pfn-dependent killing (25). Fig. 5A, right panel, demonstrates that peptide-specific killing by CD4 effectors was inhibited by as little as 100 nM CMA, whereas 500 nM CMA inhibited 60–70% of lysis at an E:T ratio of 10:1, suggesting that CD4 effectors could use Pfn as a mechanism of cytolytic activity.

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Discussion

Our studies evaluated the functional potential of primed CD4 T cells against a highly pathogenic strain of influenza virus and demonstrated that CD4 effectors could prevent mortality using distinct mechanisms. In vitro-generated CD4 effectors, adoptively transferred into unprimed hosts, mediated protection independently of donor IFN-γ and host T cells. CD4 effectors seemed to have direct effects in the lung that resulted in decreased viral titers in the first week of infection. Furthermore, protection mediated by CD4 effectors was associated with accelerated and enhanced anti-influenza Ab production. Although CD4 effectors delayed death in B cell-deficient mice, convalescent immune sera administered 7 days postinfection was required to sustain survival at high doses of pathogenic virus. It was also demonstrated that in vitro-generated CD4 effectors acquired Pfn-mediated cytolytic activity that was retained in vivo. Pfn-deficient CD4 cells were less effective than WT cells in promoting survival to high doses of PR8 in normal BALB/c mice and did not provide protection in B cell-deficient mice, indicating that Pfn was required for Ab-independent protection mediated by CD4 effectors. Therefore, distinct mechanisms worked synergistically to prevent mortality to supralethal infection: Pfn-dependent cytotoxicity acted early in the response in a B cell-independent manner, whereas CD4-driven Ab production was involved later in the response and neutralized remaining pathogenic virus.

In the wake of recent reports detailing the possible spread of avian influenza strains and the current vaccine strategies to combat such pandemic viruses (4), understanding the mechanisms involved in cell-mediated or heterosubtypic immunity has become paramount (43). Previous studies investigating the importance of primed CD4 T cells against lethal infection were done with repeatedly stimulated T cell clones and suggested a role for B cell-dependent and -independent pathways in promoting survival (14, 44). Furthermore, Graham et al. (16) demonstrated that CD4 cell clones deficient in IFN-γ secretion could mediate protection against lethal infection with the less pathogenic A/Japan/57 strain of influenza, although the mechanisms involved were not further determined. Our study extends these earlier results to show that CD4 effectors generated by a primary stimulation are effective against high doses of an extremely pathogenic strain of influenza A. We demonstrate here that CD4 effectors could provide protection independent of IFN-γ secretion, even over a wide range of influenza challenge doses (Fig. 3). In addition, we found that IFN-γ was not required, during the priming phase, for the generation of protective CD4 effectors (Fig. 2). Indeed, IFN-γ−/− effectors generated in the presence of B cell blasts from IFN-γ−/− mice could also protect normal BALB/c mice from lethal infection (data not shown). These results, as well as data obtained using IFN-γ-deficient mice as recipients (data not shown), clearly demonstrate that IFN-γ is not required for CD4-mediated protection to influenza.

Surprisingly, effectors deficient in IFN-γ secretion promoted survival better than WT effectors, especially in the presence of convalescent serum. In our experiments, B cell-deficient mice in promoting survival at 10,000 and 20,000 EIU PR8; however, 60% of mice given Pfn−/− effectors still survived a very high (~10 LD50) dose of PR8. Although this difference did not reach statistical significance, the interpretation of this result was complicated by the Ab response in intact BALB/c mice. Therefore, experiments were repeated in B cell-deficient J1gD mice at a dose of virus where CD4-mediated protection is Ab independent. Fig. 6D shows the weight loss and survival of J1gD mice injected with WT or Pfn−/− effectors and subsequently infected with 1,500 EIU PR8 virus. As seen in Fig. 5C, J1gD mice given WT CD4 effectors lost weight until about day 5, at which point, weight loss was reversed, and mice regained their body weight by day 15 postinfection. In contrast, mice given Pfn−/− effectors rapidly lost body weight, did not demonstrate a plateau in weight loss, and eventually succumbed to lethal infection. These data indicate that Ab-independent, CD4-mediated protection to lethal influenza infection requires Pfn, and suggest that CD4 effectors may mediate direct cytotoxicity at the site of lethal infection.
given IFN-\(\gamma^{--}\) effectors had an increased mean time to death and an increased survival when given suboptimal amounts of convalescent sera (Fig. 4, C and E). The ability of IFN-\(\gamma^{--}\) effectors to promote better survival correlated with higher cytolytic activity and a slightly enhanced ability to promote anti-influenza Ab responses when compared with WT cells (Fig. 5A). Interestingly, IFN-\(\gamma^{--}\) cells also produced higher levels IL-4, IL-5, and IL-17 in comparison to WT effectors. It has been demonstrated that the production of Th2 cytokines (IL-4, IL-5) is detrimental to the host during lethal influenza infection (14, 45); however, the role of IL-17 in CD4-mediated protection has yet to be determined. Overall, our data suggest that IFN-\(\gamma\) production by primed CD4 cells is not a good correlate of protection against influenza, whereas Pfn-dependent cytolytic activity and ability to help induce Ab production are better prognostic indicators.

CD4-mediated protection to very high doses of lethal PR8 virus relied on the presence of B cells and their ability to produce neutralizing Abs that have therapeutic activity upon transfer. CD4 effectors induced a more rapid Ab response, resulting in high anti-influenza IgG1 and IgG2a Ab titers in BALB/c mice challenged with a lethal dose of influenza, suggesting that a major mechanism of CD4-mediated protection involves the ability of CD4 effectors to act as helper cells. The importance of this mechanism is illustrated in B cell-deficient mice, where CD4 effectors could not promote complete survival to a lethal dose of 2500 EIU PR8 virus. Two different groups studying protection to primary influenza infection found that B cell-deficient mice, depleted of CD8 T cells, could not combat what would normally be a sublethal dose in intact mice. In those experiments, mice could not clear PR8 (46) or X31 (47) influenza virus and demonstrated a higher mortality rate, suggesting that naive CD4 cells alone could not promote survival to a highly pathogenic influenza virus. This parallels what we observed when naive HA-specific TCR Tg CD4 cells were transferred, but did not confer protection in BALB/c mice (Fig. 1B). We postulate that naive CD4 T cells cannot develop into primed effectors quickly enough to combat the exponential increase in replicating virus at such high doses of influenza.

In another study of primary protection, B cell-deficient mice that are also depleted of CD4 cells demonstrate high mortality to an otherwise sublethal dose in normal mice (48), again implying that it is the interaction between CD4 cells and B cells that is important for controlling pathogenic influenza virus infection. In fact, the data shown in Fig. 4 would suggest that survival in intact BALB/c mice depends upon the ability of CD4 effectors to drive the differentiation of influenza-specific B cells, resulting in the production of high neutralizing Ab titers. We found that primed CD4 effectors were also able to control infection in B cell-deficient mice when viral doses were lower (Fig. 6C), but that protection to high levels of PR8 virus required both primed CD4 cells and either B cells or passive Ab. Throughout our studies, it is clear that initial viral dose has a profound effect on the need for CD4 T cell priming and for synergy between the two mechanisms of protection we have identified; such that, as viral dose increases, the need for priming and the Pfn-dependent component becomes more apparent (Fig. 6, C and D).

The premise that CD4 effectors promote survival by enhancing B cell Ab generation is strengthened by our observation that convalescent sera, in conjunction with CD4 effectors, provides protection in B cell-deficient mice. A recent report from Mozdzanowska et al. (49) group also demonstrated that the efficacy of passive Ab transfer was enhanced in the presence of endogenous CD4 cells in a primary response to influenza. In that study, highly purified mAb was injected before infection, and several times thereafter, to promote survival, but the contribution of the CD4 component was not elucidated. Importantly, our results demonstrate that therapeutically administered convalescent sera can synergize with primed CD4 cells to provide protection to high viral doses in B cell-deficient mice, even when addition of serum is delayed for 1 wk. Convalescent sera alone could not rescue J\(\beta\)D mice, but CD4 effectors alone could lengthen median survival time and abrogate early weight loss in B cell-deficient mice. Taken together, these data suggest that CD4 effectors can confer some level of protection in the early phase of lethal infection in unprimed hosts. In fact, at lower doses of lethal PR8 virus, an Ab-independent role for CD4 effectors was revealed (Fig. 5C). In contrast to previously published results (44, 46, 47), our data indicated that primed CD4 cells, in the absence of B cells, could confer protection to lethal influenza infection. It should be noted that in our studies, CD4 cells did not prevent infection, or inhibit clinical disease, but did act to reduce disease severity and prevent mortality.

The acquisition of Pfn-mediated cytolytic activity by CD4 effectors suggests that this mechanism of cell killing is important in peptide-specific responses in normal, immune competent mice. Previous reports have demonstrated that killing by CD4 cells was attributed to the Fas:FasL pathway (39), occurred in response to transformed cells (26) or MLRs (50), and was best identified in mice that lacked the conventional CD8 cytolytic compartment (51). In addition to earlier work by Graham et al. (16) and Lukacher et al. (52) using CD4 cell clones, our results demonstrate that primary CD4 effectors, generated by only 4 days in culture, are highly cytolytic to peptide-pulsed targets in vitro, use Pfn as the major mechanism of cell lysis, and retain this cytolytic activity in vivo. It is interesting to note that CD4 effectors deficient in IFN-\(\gamma\) also acquired Pfn-mediated cytolyis and consistently displayed higher activity than WT effectors (Fig. 5). A seeming paradox is the fact that Th2-polarized cells demonstrate less cytolytic activity and do not provide complete protection to lethal influenza infection (14) (D. M. Brown, unpublished data), suggesting a correlation between Th1 polarization and cytolytic activity, but one that does not require IFN-\(\gamma\). Using this model of in vitro effector generation, we should be able to define the conditions and decipher the signals that drive the development of Pfn-mediated cytotoxicity in CD4 T cells.

There have been many reports describing the cytolytic potential of class II-restricted CD4 cells, but the precise role of these cells in viral clearance has not been investigated. In humans, cytolytic CD4 cells have been identified in patients with HIV and EBV (34) and in mice exposed to lymphocytic choriomeningitis virus (33). Although CD4 cells can acquire lytic activity, the availability of MHC class II-expressing cells may be a limiting factor in CD4-mediated killing in our model. Previous experiments using bone marrow chimaeras demonstrated that CD4 cells could clear influenza virus from a class II\(^{--}\) respiratory tract (53), suggesting that CD4-mediated killing was not required for viral clearance, and that Ab-mediated pathways contributed to viral clearance. This is not to suggest, however, that direct recognition by cytolytic CD4 effectors and elimination of class II\(^{--}\)-infected epithelial cells does not contribute to protection. In humans, epithelial cells have been shown to express class II molecules constitutively (54, 55), and many lung injuries and infections can further induce class II MHC molecules on the lung epithilia (56, 57). In experimental influenza infection of mice, virus can be detected in both the bronchial and alveolar epithelial cells (45), thus increasing the possibility of viral peptides being processed and presented via class II molecules on infected epithelial cells and these cells acting as targets for...
CD4-mediated cytotoxicity. Clearly, the ability of CD4 effectors to acquire Pfn-mediated cytotoxicity is an important component of protection in normal responses, and it may be crucial in an Ab-independent, or a B cell-deficient, environment. Cytotoxicity by primed CD4 cells may contribute to lowering viral burden, especially in situations involving high doses of virus or highly pathogenic strains of influenza. This Pfn-mediated mechanism may be important early in the protective response by controlling viral titers in the lung until enough neutralizing Abs are generated to clear remaining virus. It remains to be determined whether cytolytic CD4 cells are generated during a sublethal dose of influenza, and studies are underway in our laboratory to further elucidate the role of cytolytic CD4 cells in vivo.

Using TCR Tg cells and in vitro-primed CD4 effectors has provided a framework for further investigation of CD4 effector functions and an understanding of the complex interactions that occur in vivo in response to lethal infection. Moreover, these results stress that, what appears to be a fairly homogeneous population of CD4 effectors can mediate both conventional helper activity and, less expectedly, cytotoxic function. It is not clear whether these distinct functions are mediated by two different subsets of CD4 effector cells within the population, or if the same cell can provide both functions in vivo. One provocative hypothesis is that the CD4 effectors have the potential to be both cytolytic and provide B cell help, depending upon where that cell localizes during the response and what host cell it encounters. For example, CD4 effectors that migrate to the lung after transfer would be able to exert cytotoxic activity when recognizing an infected epithelial cell, while the same effectors that remain in the LN migrate to germinall centers and provide help when interacting with an activated Ag-presenting B cell. Using the in vitro system, CD4 effectors can be generated that either lack cytolytic activity or lack helper function to further elucidate these mechanisms.

Finally, although CD4 effectors primed in vitro can provide protection to lethal influenza infection, it is important to demonstrate that CD4 effectors primed in vivo can also promote survival. Our preliminary data suggest that CD4 effectors isolated from draining LN or lung during a sublethal infection with PR8 can provide protection upon adoptive transfer to lethally infected mice (D. M. Brown, manuscript in preparation). Further studies are needed to elucidate the mechanisms used by in vivo-primed CD4 effectors in mediating protection; however, we speculate that the ability to help the B cell response as well as a cytolytic component will be important factors in this model as well. We also hypothesize that long-term memory CD4 cells will provide protection by similar mechanisms. Transferred effectors quickly revert to “rested effectors” that display memory CD4 activity (58), and our preliminary results indicate that effectors rested in vitro, which are nearly identical with those rested in vivo, are as protective as 4-day effectors (D. M. Brown, unpublished observations).

Deciphering the signals important in the generation of multifunctional CD4 effectors will provide crucial information for vaccine design. Current vaccines to influenza virus exploit the humoral response and target the highly variable outer surface proteins of the virus (4); however, these vaccines are strain specific and may be ineffective against a new variant of influenza that has serologically distinct coat proteins (43). Therefore, in the event of mutation of H5N1 avian influenza, the general population is unlikely to have effective immunity to the highly variable outer surface proteins of the virus. However, because internal core proteins are highly conserved, vaccine strategies to prime heterosubtypic immunity would likely result in primed T cells that are able to respond to emerging influenza by targeting the conserved internal proteins such as nucleoprotein and PA (43). Based on our data, priming CD4 responses with vaccines such as live, attenuated virus, could synergize with unprimed B cells to promote some level of protection against an H5N1 virus that would share internal epitopes. In summary, our data underscore the ability of cytolytic CD4 effectors to provide protection via an Ab-independent mechanism and support the potential of heterosubtypic vaccine strategies that may protect against lethal forms of virus.

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Disclosures
The authors have no financial conflict of interest.

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