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The Magnitude of the T Cell Response to a Clinically Significant Dose of Influenza Virus Is Regulated by TRAIL

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An immune response of appropriate magnitude should be robust enough to control pathogen spread but not simultaneously lead to immunopathology. Primary infection with influenza A virus (IAV) results in a localized pulmonary infection and inflammation and elicits an IAV-specific CD8 T cell immune response necessary for viral clearance. Clearance of IAV-infected cells, and recovery from infection, is mediated by perforin/granzyme B- and Fas/FasL-mediated mechanisms. We recently reported that TRAIL is another means by which IAV-specific CD8 T cells can kill IAV-infected cells. The current study examined the role of TRAIL in the pulmonary CD8 T cell response to a clinically significant IAV [A/PR/8/34 (PR8; H1N1)] infection (i.e., leads to observable, but limited, morbidity and mortality in wild-type [WT] mice). Compared with WT mice, IAV-infected Trail™/ mice experienced increased morbidity and mortality despite similar rates of viral clearance from the lungs. The increased morbidity and mortality in Trail™/ mice correlated with increased pulmonary pathology and inflammatory chemokine production. Analysis of lung-infiltrating lymphocytes revealed increased numbers of IAV-specific CD8 T cells in infected Trail™/ mice, which correlated with increased pulmonary cytotoxic activity and increased pulmonary expression of MIG and MIP-1α. In addition, there was decreased apoptosis and increased proliferation of IAV-specific CD8 T cells in the lungs of Trail™/ mice compared with WT mice. Together, these data suggest that TRAIL regulates the magnitude of the IAV-specific CD8 T cell response during a clinically significant IAV infection to decrease the chance for infection-induced immunopathology. 


Primary infection with influenza A virus (IAV) results in a localized pulmonary infection that induces an IAV-specific CD8 T cell immune response essential for efficient viral clearance (1–8). Recruitment of IAV-specific T cells into the lung after their initial priming in lung-draining lymph nodes (LN) is dependent on chemokine expression in the lung. A plethora of chemokines have been implicated in T cell homing to airway after IAV infection, including MIP-1α, MIP-1β, MIP-3α, RANTES, and IP-10 (9–13). Similarly, expression of the corresponding chemokine receptors—including CCR2, CCR5, and CXCR3—has been detected on activated, IAV-specific T cells after IAV infection. The interactions of these chemokines with their respective receptors enhance integrin expression, which is essential for IAV-specific T cell migration into the lung environment (9, 13–16).

The initiation of the T cell response to primary IAV infection also leads to the expression of effector molecules (such as FasL and perforin/granzyme B) used by the T cells to kill IAV-infected cells (7). In addition to these well-characterized cytotoxic pathways, previous work from our laboratory identified a role for TRAIL-expressing CD8 T cells in the primary immune response to IAV infection (17). TRAIL has classically been studied in tumor immunology settings because it selectively induces apoptosis in transformed cells while leaving normal cells and tissues unaffected (18–20), but TRAIL-based immunity is becoming more appreciated as a key component in the immune response to viral infections—including responses to CMV, HIV, and respiratory syncytial virus (21–28). Although TRAIL-expressing IAV-specific CD8 T cells participate in the killing of virally infected cells (17), TRAIL expression on alveolar macrophages has also been associated with increased lung damage and susceptibility to IAV pneumonia (29, 30). Thus, depending on the pathogenicity of the virus or the size of the initial inoculum, TRAIL may have beneficial or detrimental roles in the immune response to IAV.

Seasonal IAV infections affect 10–20% of the U.S. population each winter, resulting in substantial morbidity (~114,000 hospitalizations) and mortality (~36,000 deaths) (31). Using an experimental model that mimics the typical infection characteristics of humans is imperative to understanding the properties of the immune response that result in IAV clearance while limiting immunopathology. The infectious dose of IAV [A/PR/8/34 (PR8; H1N1)] used in our previous investigation did not induce mortality in either wild-type (WT) or Trail™/ mice, but it did result in significant morbidity in the Trail™/ mice (17). In the current study, we altered our infection protocol to examine the role of TRAIL in the CD8 T cell-mediated immunity induced during...
a clinically significant IAV infection that results in limited morbidity and mortality in WT mice. Our results suggest that TRAIL plays a significant role in limiting the magnitude of the IAV-specific CD8+ T cell response in the lungs during a clinically significant IAV infection through alterations in IAV-specific CD8+ T cell recruitment to the site of infection as well as the proliferation and survival of these T cells in the lung.

Materials and Methods

Mice, virus, infections, and peptides

WT C57BL/6 (B6) and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively. Trail−/− B6 and BALB/c mice were obtained from Amgen (Seattle, WA) (32) and Dr. Thomas Sayers (National Cancer Institute, Frederick, MD), respectively. Both strains of Trail−/− mice have been back-crossed onto the B6 or BALB/c background ≥10 generations. CXCR3−/− mice were obtained from Dr. Steven Varga (University of Iowa). All mice were used at 16–24 wk of age, and all experiments followed approved University of Iowa Institutional Animal Care and Use Committee protocols. The mouse-adapted A/PuertoRico/8/34 (PR8; H1N1) and X-31 (H3N2) IAV strains were grown in the allantoic fluid of 10–d-old embryonated chicken eggs for 2 d at 37°C, as previously described (5, 33, 34). Allantoic fluid was harvested and stored at −80°C. Groups of 20–22.5 WT and Trail−/− mice were given 1500 egg-infectious units (EIU) of mouse-adapted PR8 or X3-1 in isovec’s media intranasally as anesthesia with isofluran. The IAV peptides NP366 (ASNENMETM and PA224 (SSLNFRAYV)) for B6 and HA322 (IYSTVAGSL) and NP368 (TIQR-TRAILV) for BALB/c were purchased from Biosynthesis (Lewisville, TX). IAV tetramer-purified spleenocytes (105 PFU) were 2D6-520 stabilized (2D6-FRAYV) for B6 and HA322 (H-2Kk/IYSTVAGSL) and NP367 (H-2Kk/TYQR-TRIALV) for BALB/c were obtained from the National Institutes of Health Tetrramer Core Facility (Germantown, MD).

Determination of lung virus titer

Pulmonary IAV titers were determined via endpoint dilution assay and expressed as tissue culture-infective dose 50% (TCID50) as previously described (35). Briefly, 10-fold dilutions of homogenized and clarified lung from IAV-infected mice were mixed with 105 Madin–Darby canine kidney cells in DMEM. After 24 h incubation at 37°C, the inoculum was removed, and DMEM containing 0.0002% L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington Diagnostics, Freehold, NJ) and penicillin (100 U/ml)/streptomycin (100 μg/ml) was added to each well. After 3 d incubation at 37°C, supernatants were mixed with an equal volume of 0.5% chicken red blood cells, the agglutination pattern read, and the TCID50 values calculated.

Histology

Whole lungs with the heart attached were harvested from WT or Trail−/− mice on various days after IAV infection. Lungs were inflated and placed in 10% formalin. After 10 d fixation, lungs were processed and embedded in paraffin. Five-micrometer-thickness sections were stained with H&E or stained with anti-CD3ε (clone Leu-4; BD Biosciences), anti-CD8 (clone H12-8C5; obtained from W. Gerhard, Wistar Institute, Philadelphia, PA) and counterstained with hematoxylin. The identity of the slides was blinded, and the slides were then scored by a board-certified veterinary pathologist. The intensity of the cellular infiltration in the H&E-stained slides was scored as follows: 0, none detected; 1, rare to uncommon; 2, detectable extravasated neutrophils in small aggregates in airway and/or alveoli; 3, multiple moderate foci/aggregates in airway and/or alveoli; and 4, severe coalescing foci/aggregates that efface alveoli.

Cytotoxicity assay

In vivo. Splenocytes were resuspended in Nycodenz 1.077 A (Axis-Shield, Norton, MA) and then purified according to the manufacturer’s instructions. Nycodenz-purified splenic mononuclear cells (107/ml) were mixed with either 2 μM CFSE (Invitrogen, Eugene, OR) at 37°C for 10 min or 2 μM PKH-26 (Sigma, St. Louis, MO) at room temperature for 5 min. After labeling, residual non-cell-associated CFSE and PKH-26 were neutralized by adding an equal volume of FCS to the cell suspension. CFSE-labeled splenic mononuclear cells (109/ml) were similarly incubated but without peptide. The cells were then washed and mixed at a 1 pulsed:1 unpulsed ratio, and 107 cells were adoptively transferred i.v. into WT or Trail−/− mice 8 d after IAV infection. After 8 h, the lungs were removed, digested, and analyzed by flow cytometry, as previously described (5), to enumerate the number of remaining target cells. Uninfected mice were used as controls. The reduction in the number of recovered peptide-pulsed target cells in the IAV-infected versus uninfected mice was considered the percent specific lysis.

In vitro. On day 8 after IAV infection, lungs were harvested from WT or Trail−/− mice, homogenized, and CDF1 T cells were MACS purified (>95% purity). A portion of the purified T cells was then stained with anti-CD8α, NP368 tetramer, and PA224 tetramer. The percentage of tetramer+ CDF1 T cells was used to calculate the number of IAV-specific effectors. For each IAV strain, B6 splenocytes were prepared and pulsed with NP366 and PA224 peptide for 1 h at 37°C as described earlier. The cells were then labeled with 100 μCi 51Cr for 1 h at 37°C, washed three times, and resuspended in complete medium. The effector CDF1 T cells were mixed with the peptide-pulsed target cells at a 50:1 E:T ratio and cultured for 18 h in a 96-well round-bottom plate. The percent specific lysis was calculated as: 100 × (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm). Spontaneous and total 51Cr release was determined in the presence of either medium alone or 1% Nonidet P-40, respectively.

Flow cytometry

Quantitation of IAV-specific CD8 T cells was performed as follows. Lungs were harvested from IAV-infected WT or Trail−/− mice. The isolated cells were then stained with FITC-conjugated anti-CD3ε (145-1 clone; eBioscience, San Diego, CA), PerCP–Cy5–conjugated anti-CD8α (53-6.7; eBioscience), and either allophycocyanin-conjugated NP366 or PA224 tetramer. The number of CD8+ T cells from the infected WT or Trail−/− mice was enumerated using total pulmonary cell counts and flow cytometry.

Surface labeling. In addition to the above mAb and tetramers, isolated lung cells were stained with PE-conjugated anti-mouse CXCR3 (CXCR3-173; eBioscience), or CCR5 (HM-CCR5; eBioscience) mAb. To examine FasL expression, cells were first blocked with 1:100 rat and hamster serum as well as unconjugated streptavidin (Molecular Probes, Eugene, OR). Cells were washed and then stained with biotinylated FasL (MFL3; eBioscience), followed by streptavidin PE. Stained cells were fixed and erythrocytes lysed with FACS lysing solution (BD Biosciences) and subsequently analyzed on a FACS Calibur flow cytometer.

Intracellular staining. Granzyme B: Isolated lung cells (106) were surface stained with anti-CD3ε, anti-CD8α, and NP366 or PA224 tetramer as described earlier. Subsequently, the cells were fixed, permeabilized, and stained with the PE-conjugated anti-human granzyme B mAb (GB11; Invitrogen), or isotype control. IFN-γ/TNF. Isolated lung cells were cultured at 2 × 106 cells/well in a 24-well plate in the presence of 1 μM of either NP366 or PA224 peptide or media control, 400 U/ml recombinant human IL-2, and 1 μg/ml brefeldin A. After 6 h, the cells were surface stained with PE-conjugated anti-mouse CD8α, fixed, permeabilized, and stained with allophycocyanin-conjugated anti-human mouse ε (XM99, eBioscience) and either FITC-conjugated anti-mouse TNF (MPS-XT22; eBioscience) or isotype control. CD107a: Isolated lung cells were cultured with NP366 or PA224 peptide, IL-2, and brefeldin A as described earlier, as well as FITC-conjugated anti-CD107a (1D45; eBioscience) or isotype control. After 6 h, cells were surface stained with PE-conjugated anti-mouse CD8α, fixed, permeabilized, and stained with allophycocyanin-conjugated anti-mouse IFN-γ or isotype control as above. Caspase 3/7: The FAM caspase 3/7 kit (Vivantis) was purchased from Invitrogen and used according to the manufacturer’s instructions. BrdU: WT and Trail−/− mice were infected with 1500 EIU PR8 as above. On day 7 postinfection (p.i.), the lung resident cells were initially labeled by intranasal CFSE administration (33, 36). Two hours after CFSE administration, the mice were given 0.8 mg/mouse BrdU intranasally. After another 4 h, the number of BrdU+ NP366 or PA224 tetramer+ T cells was measured by flow cytometry (36). All flow cytometry data were acquired on an LSR II (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Measurement of pulmonary chemokines

Lungs were harvested from IAV-infected WT or Trail−/− mice on day 6 p.i. and homogenized in 3 ml DMEM. Subsequently, the pulmonary chemokine expression was determined using a mouse chemokine array (Invitrogen, Carlsbad, CA).

Statistical analysis

For each analysis, normal distribution of data was first verified. To assess the difference between two sets of data with normal distribution, statistical significance was assessed using an unpaired, one-tailed t test or a paired t test for control and experimental data groups that could be paired. If normality test failed, Mann–Whitney rank sum tests were completed to compare data.
sets. To assess the differences among multiple sets of data with normal distribution, statistical significance was assessed using an ANOVA analysis of the data sets. If normality test failed, Kruskal–Wallis one-way ANOVA on ranks test was used to determine overall significance with subsequent pairwise comparisons completed using Dunn’s method. To determine differences in interstitial inflammation p.i., a Wilcoxon two-sided two-sample exact test was run. When appropriate, subsequent pairwise multiple comparisons were completed using the Holm–Sidak method. Differences were considered to be statistically significant at \( p \leq 0.05 \).

**Results**

Trail \(^{-/-}\) mice exhibit increased morbidity and mortality during a clinically significant IAV infection compared with WT mice

Our previous report examining the role of TRAIL in the immune response to IAV infections used a subclinical dose of IAV that induced minimal morbidity and no mortality in WT mice (17). Although it is possible for humans to experience asymptomatic IAV infections, there is significant public health interest in better understanding the primary immune response to IAV infections that result in the development of clinical symptoms. Thus, we altered the infectious inoculum of the high virulent IAV strain PR8 and starting animal weight to induce observable morbidity in WT B6 mice (Fig. 1A). In this setting, Trail \(^{-/-}\) B6 mice showed significantly increased weight loss on days 6–10 p.i. compared with WT B6 mice that correlated with significantly increased mortality (Fig. 1B). Reinforcing the importance for TRAIL in the immune response to IAV infection, increased mortality was also observed in Trail \(^{-/-}\) BALB/c mice given a clinically significant IAV compared with WT BALB/c mice (Supplemental Fig. 1).

Another aspect from our earlier studies with the subclinical IAV infection was a significant increase in lung viral titers and a significant delay in viral clearance in Trail \(^{-/-}\) B6 mice compared with WT B6 mice (17). Because the IAV infection itself can cause some pathology, we examined the extent to which the above differences in morbidity and mortality were related to any differences in viral clearance. Despite the observed increased morbidity and mortality in Trail \(^{-/-}\) B6 mice, the IAV titers and rate of viral clearance from the lungs of WT and Trail \(^{-/-}\) B6 mice were surprisingly similar (Fig. 1C). These data suggest the increased morbidity and mortality in Trail \(^{-/-}\) B6 mice after a clinically significant IAV infection was not due to an increased pulmonary viral load.

**Trail \(^{-/-}\) mice have increased pulmonary inflammation compared with WT mice during a clinically significant high virulent IAV infection**

The extent of lung injury in the context of IAV clearance is influenced by viral load and the magnitude of the IAV-specific CD8 T cell response. For example, when the viral load is low, CD8 T cells can potently limit IAV replication and protect IAV-induced lung injury, but significant immunopathology can occur when the IAV load is high (37). The efficiency with which Trail \(^{-/-}\) B6 mice cleared the clinically significant IAV infection, combined with the increased morbidity and mortality of these mice, prompted us to examine the lungs histologically. Consistent with the more extensive morbidity and increased mortality in Trail \(^{-/-}\) B6 mice, histological evaluation of the lung revealed significantly increased interstitial inflammation in the Trail \(^{-/-}\) B6 mice after IAV infection (Fig. 2A, 2B). This increase in inflammation in the Trail \(^{-/-}\) B6 mice correlated with increased pulmonary tissue damage and loss of organized tissue architecture. These data suggest the enhanced morbidity and mortality in Trail \(^{-/-}\) B6 mice after clinical dose IAV infection may result from increased inflammation and immune-mediated tissue damage.

**Trail \(^{-/-}\) mice have enhanced pulmonary recruitment of IAV-specific CD8 T cells during a clinically significant IAV infection compared with WT mice**

The clearance of IAV from the lung, as well as the increased mortality of Trail \(^{-/-}\) B6 mice, is consistent with the kinetics of CD8 T cell infiltration into the lungs after IAV infection (4, 5). During primary IAV infections, CD8 T cells contribute to viral clearance (6, 38) by triggering apoptosis in the infected cells through FasL–Fas interactions, perforin/granzyme B secretion (7), and TRAIL–DR5 interactions (17). Our previous analysis of subclinical IAV infections revealed that inefficient viral clearance in Trail \(^{-/-}\) B6 mice was attributed to decreased cytotoxic function by IAV-specific CD8 T cells (17). The efficiency with which Trail \(^{-/-}\) mice cleared a clinically significant IAV infection prompted us to measure IAV-specific CD8 T cell cytolytic function. To our surprise, the in vivo cytotoxic activity of IAV-specific CD8 T cells was significantly higher in Trail \(^{-/-}\) mice versus WT B6 mice (Fig. 3A). In contrast, the in vitro killing activity of IAV-specific CD8 T cells from the lungs of infected WT or Trail \(^{-/-}\) B6 mice was nearly identical when measured on a per-cell basis (Fig. 3B). To determine the extent to which a change in the effector phenotype of the Trail \(^{-/-}\) IAV-specific CD8 T cells responding to the infection contributed to the observed cytotoxic difference, we examined the expression of several cytotoxic proteins known to be critical for the cytotoxic function of IAV-specific T cells. Phenotypic analysis of the IAV-specific CD8 T cells showed no significant difference in the expression of FasL, granzyme B, and TNF and had a similar ability to degranulate, as measured by CD107a staining (Fig. 3C, 3D).

**FIGURE 1.** TRAIL deficiency correlates with increased disease severity during a clinically significant IAV infection. A and B, WT or Trail \(^{-/-}\) B6 mice \((n = 6 \text{ mice/group})\) were infected intranasally with 1500 EIU A/PR/8/34 and weighed daily to assess morbidity (A) and mortality (B). In A, the values displayed represent the daily weight relative to the weight on day of infection. In B, data represent the percentage of mice surviving on the given day p.i.; significantly increased mortality was observed in the Trail \(^{-/-}\) mice. Data are representative of two separate experiments. C, Given a clinically significant IAV infection \((1500 \text{ EIU} \text{ A/PR/8/34})\), WT and Trail \(^{-/-}\) mice have similar viral titers and clearance. At indicated days p.i., lungs were harvested, and pulmonary viral titers were assessed by determining the TCID\(_{50}\) in Madin–Darby canine kidney cell cultures. No significant difference was observed in the viral titers or the rate of viral clearance at the clinical dose of infection. Data are representative of two separate experiments with three to five mice per group.
Having observed minimal phenotypic differences between the IAV-specific WT and Trail^2^/2^ CD8 T cells on a per-cell basis, we quantitated the magnitude of the IAV-specific T cell response to determine the extent to which T cell numbers might have contributed to the enhanced in vivo pulmonary T cell cytotoxicity in Trail^2^/2^ B6 mice. This analysis revealed a significant increase in the number of NP366- and PA224-specific CD8 T cells in the lungs of Trail^2^/2^ versus WT B6 mice (Fig. 4A). Consistent with these findings, analysis of pulmonary HA529- and NP147-specific CD8 T cells in WT and Trail^2^/2^ BALB/c mice showed similar

**FIGURE 2.** Trail^2^/2^ mice have increased pulmonary cellular infiltration and increased inflammation during a clinically significant IAV infection. WT or Trail^2^/2^ B6 mice (n = 6 mice/group) were infected with 1500 EIU A/PR/8/34. On various days p.i., lungs were harvested and insufflated with 10% buffered formalin. A. Subsequently, the lung tissue was sectioned, mounted, and stained with H&E (original magnification X10). B. The identities of the slides were blinded, and slides were evaluated; scores for each time point are indicated. Averaged results are presented, and statistical comparisons between WT and Trail^2^/2^ mice were done using the Wilcoxon two-sided two-sample exact test. *p < 0.05.

**FIGURE 3.** Trail^2^/2^ mice display enhanced IAV-specific CD8^+^ T cell-mediated in vivo cytotoxicity compared with WT mice, despite similar cytotoxic molecule expression. A. The pulmonary IAV-specific CD8 T cell response in WT or Trail^2^/2^ B6 mice infected with 1500 EIU A/PR/8/34 was measured by in vivo cytotoxicity assay on day 8 p.i. Symbols represent killing in individual mice, and bars represent mean killing. Percentage IAV-specific killing was calculated by comparing unpulsed target lysis to IAV peptide-pulsed target lysis. Target cells were verified to be DR5^+^ by flow cytometry (data not shown), and target cell frequencies were normalized to ratios harvested from transfers into naive mice. B. In contrast to the in vivo cytotoxicity, the in vitro cytotoxic activity of WT and Trail^2^/2^ IAV-specific CD8 T cells was similar. IAV-specific CD8 T cells were MACS-purified from the lungs of WT or Trail^2^/2^ B6 mice infected with 1500 EIU A/PR/8/34 on day 8 p.i. The T cells were then cultured with unpulsed or IAV peptide-pulsed ^51^Cr-labeled splenocytes at a 50:1 E:T ratio for 18 h. Bars represent the mean (±SD) specific lysis measured from triplicate wells. No significant (n.s.) difference was observed between groups containing WT and Trail^2^/2^ effector cells. C and D. Pulmonary T cells from WT and Trail^2^/2^ mice have similar expression of effector molecules. WT or Trail^2^/2^ B6 mice were infected with 1500 EIU A/PR/8/34, and then lungs were harvested on day 8 p.i. Isolated cells were stained with anti-CD8^a^, NP366 tetramer or PA224 tetramer, anti-CD3^ε^, anti-granzyme B or isotype control Ab, and anti-FasL or isotype control Ab. Solid-line histograms represent FasL or granzyme B staining on CD8^+^tetramer^+^ T cells. Gray histograms represent isotype control staining. For TNF and CD107a analysis (D), isolated cells were incubated with NP366 or PA224 peptides or control media, brefeldin A, and anti-CD107a for 5 h. After incubation, the cells were stained with anti-CD8^a^, anti–IFN-γ or isotype control Ab, and anti-TNF or isotype control Ab. Solid-line histograms represent TNF or CD107a expression on CD8^+^IFN-γ^+^ cells. Gray histograms represent isotype control staining.
with the low virulent IA V strain, X-31 (Supplemental Fig. 3), isolated cells were stained with anti-CD3 days 6, 8, and 10 p.i., lungs were harvested and homogenized, and the cells were stained with anti-CD8. Lungs were harvested on day 8 p.i., and the isolated cells were incubated in vitro with NP336 or PA224 peptide for 6 h. The number of Ag-specific CD8 T cells, based on IFN-γ/Trail or PA224 tetramer, anti-CD3/ε, and NP366 tetramer/ε production after restimulation, from the infected WT or Trail−/− mice was enumerated using total pulmonary cell counts and flow cytometry. Data are averaged from five mice per group. B, On day 8 p.i., lungs were harvested and homogenized, and the isolated cells were incubated in vitro with NP336 or PA224 peptide for 6 h. The number of Ag-specific CD8 T cells, based on IFN-γ production after restimulation, from the infected WT or Trail−/− mice was enumerated using total pulmonary cell counts and flow cytometry. Data are averaged from five mice per group.

increases in the IAV-specific CD8 T cell response (Supplemental Fig. 2). The increase in the IAV-specific CD8 T cell response in Trail−/− B6 mice was also seen when determining the number of IFN-γ-producing CD8 T cells after Ag-specific in vitro restimulation (Fig. 4B). Notably, increased numbers of IAV-specific CD8 T cells were also detected in the lungs of Trail−/− B6 mice p.i. with the low virulent IAV strain, X-31 (Supplemental Fig. 3), suggesting that the exaggerated CD8 T cell response in Trail−/− mice may be independent of the IAV strain used for infection. Together, these data suggest that the increased in vivo cytotoxicity of pulmonary IAV-specific CD8 T cells in Trail−/− mice given a clinically significant IAV infection likely results from a difference in the pulmonary environment that leads to greater numbers of IAV-specific CD8 T cells in the lungs of Trail−/− mice compared with WT mice.

Trail−/− mice have increased pulmonary chemokine expression during a clinically significant IAV infection compared with WT mice

The increase in IAV-specific CD8 T cell numbers in the lungs of Trail−/− mice given a clinically significant IAV infection could be explained by three potential mechanisms: 1) altered pulmonary chemokine expression leading to increased recruitment; 2) differential apoptosis of the T cells in the lungs; and/or 3) increased proliferation of T cells within the lung. To determine the possibility of altered chemokine expression causing enhanced recruitment of IAV-specific CD8 T cells to the lungs, we first measured the pulmonary expression of chemokines associated with T cell recruitment. We observed significantly increased expression of MIG and MIP-1α in the lungs of Trail−/− mice compared with WT B6 mice (Fig. 5A). Both of these chemokines are critical for CD8 T cell migration into the lungs during IAV infection (9–12). For chemokines to effectively act on T cells and enhance their migration into the lungs, the T cells need to express the corresponding receptor for the chemokine. Examination of the Ag-specific CD8 T cells responding to IAV infection revealed that CXCR3 (receptor for MIG) and CCR5 (receptor for MIP-1α) were similarly expressed on IAV-specific pulmonary T cells from WT and Trail−/− B6 mice (Fig. 5B).

Because of the established importance for these chemokine receptors in recruiting T cells to the lungs during IAV infection (9–12), we examined the extent to which CXCR3 and CCR5 were also required for the migration of IAV-specific CD8 T cells into the lungs of clinical dose-infected Trail−/− B6 mice (experimental design depicted in Supplemental Fig. 4). CD8 T cells were isolated from the lung-draining LNs of WT and CXCR3−/− B6 mice. After pretreatment with a CCR5 blocking mAb or isotype control, these T cells were then transferred into IAV-infected WT or Trail−/− B6 mice. The T cells lacking CXCR3 migrated to the lungs of WT or Trail−/− B6 mice ∼50% less efficiently than WT T cells,

FIGURE 4. Trail−/− mice given a clinically significant IAV infection show enhanced pulmonary T cell recruitment compared with WT mice. WT or Trail−/− B6 mice were infected with 1500 EIU A/PR/8/34. A, On days 6, 8, and 10 p.i., lungs were harvested and homogenized, and the isolated cells were stained with anti-CD3ε, anti-CD8α, and NP336 tetramer or PA224 tetramer. The number of CD8+ tetramer+ T cells from the infected WT or Trail−/− mice was enumerated using total pulmonary cell counts and flow cytometry. Data are averaged from five mice per group. B, On day 8 p.i., lungs were harvested and homogenized, and the isolated cells were incubated in vitro with NP336 or PA224 peptide for 6 h. The number of Ag-specific CD8 T cells, based on IFN-γ production after restimulation, from the infected WT or Trail−/− mice was enumerated using total pulmonary cell counts and flow cytometry. Data are averaged from five mice per group.

FIGURE 5. Trail−/− mice have increased pulmonary chemokine expression during a clinically significant IAV infection compared with WT mice. WT or Trail−/− B6 mice were infected with 1500 EIU A/PR/8/34. A, Lungs were harvested on day 6 p.i. and homogenized in 3 ml DMEM. Subsequently, the pulmonary chemokine expression was determined using multiplex analysis. Data presented are the average chemokine concentration measured from four WT or Trail−/− mice and are representative of two independent experiments. B, Lungs were harvested on day 8 p.i., and the isolated cells were stained with anti-CD8α, NP336 tetramer, PA224 tetramer, anti-CD3ε, anti-CXCR3, anti-CCR5, or isotype control. Histograms show CXCR3 and CCR5 expression on CD3+CD8+ tetramer+ T cells from WT (solid line) and Trail−/− (dashed line) mice or the isotype control (shaded histogram). Data are representative of five mice from two independent experiments. C, Abrogation of chemokine signals to T cells blocks their migration to the lung after IAV infection. Details of the experimental design are presented in Supplemental Fig. 4.
but there was only a minimal alteration in the migration of T cells that had CCR5 blocked (Fig. 5C). Loss of signal through both CXCR3 and CCR5 did not further inhibit the migration of the transferred cells to the lungs of infected WT B6 recipients, but the loss of both CXCR3 and CCR5 significantly reduced the recruitment of these cells into the lungs of infected Trail−/− B6 recipients. These data reinforce the role these receptors play in T cell migration to the lung after IAV infection and suggest that increased chemokine production in the Trail−/− pulmonary environment contributes to the enhanced number of Ag-specific CD8 T cells that are recruited into the lung to respond to a clinically significant IAV infection.

**Discussion**

Trail−/− mice have decreased apoptotic death and increased proliferation of the infiltrating IAV-specific CD8 T cells in the lung p.i. compared with WT mice

To investigate the potential impact of alterations in T cell apoptosis or proliferation affecting the magnitude of the IAV-specific T cell response in this system, the following experiments were performed. First, we determined the frequency of NP366- and PA224-specific CD8 T cells within the lungs of WT and Trail−/− B6 mice undergoing apoptosis (based on active caspase 3/7) during the course of infection. Notably, there was a significant decrease in the frequency of apoptotic NP366- and PA224-specific CD8 T cells in Trail−/− B6 mice given a clinically significant IAV infection compared with WT B6 mice (Fig. 6). These data correlate with the difference in numbers of NP366- and PA224-specific CD8 T cells in WT and Trail−/− B6 mice shown in Fig. 4A. Next, we measured the proliferative status of the NP366- and PA224-specific CD8 T cells within the lung on day 7 p.i. by sequential intranasal administration of CFSE and BrdU (36). Using this procedure, the data show that whereas there was no significant difference in the frequency of NP366- and PA224-specific CD8 T cells proliferating (based on BrdU incorporation) within the lung of the infected WT and Trail−/− mice, there were increased numbers of these populations of CD8 T cells actively proliferating in the lungs of Trail−/− B6 mice given a clinically significant IAV infection compared with WT B6 mice (Fig. 7). These results suggest that in addition to increased pulmonary chemokine expression and IAV-specific CD8 T cell recruitment, enhanced pulmonary T cell responses in Trail−/− mice given a clinically significant IAV infection also result from increased proliferation and decreased apoptosis of the IAV-specific CD8 T cells in the lungs.

**FIGURE 6.** Decreased apoptosis of IAV-specific CD8 T cells in the lungs of Trail−/− mice given a clinically significant IAV infection compared with WT mice. WT or Trail−/− B6 mice were infected with 1500 EIU A/PR8/34. On days 6, 8, and 10 p.i., lungs were harvested, and the isolated cells were analyzed by flow cytometry for apoptosis of IAV-specific CD8 T cells as measured by the frequency of active caspase 3/7 cells of the CD3+CD8+NP366 tetramer+ or CD3+CD8+PA224 tetramer+ cells. Data are averaged from four mice per group per time point.

**FIGURE 7.** Increased number of actively proliferating IAV-specific CD8 T cells in Trail−/− mice given a clinically significant IAV infection compared with WT mice. WT or Trail−/− B6 mice were infected with 1500 EIU PR8. On day 7 p.i., mice were given CFSE intranasally, followed by BrdU intranasally 2 h later. Lungs were harvested 4 h later, homogenized, and the isolated cells were analyzed by flow cytometry for proliferation of IAV-specific CD8 T cells as measured by the frequency of BrdU+ cells of the CFSE+CD8+NP366 tetramer+ or CFSE+CD8+PA224 tetramer+ cells. The gating strategy, representative plots gated on CFSE+CD8+ cells (A), and averaged data (B) based on four mice/group are shown.
increased viral load, as observed in our subclinical dose infection model. Instead, the data suggest that the increased morbidity and mortality resulted from immunopathology caused (at least in part) by the recruitment of significantly increased numbers of IAV-specific CD8 T cells.

The immune response to a primary IAV infection is composed early by innate immune cells (neutrophils, NK cells, macrophages, γδ T cells), which are followed by IAV-specific CD4 and CD8 T cells and finally Abs. CD8 T cells eliminate IAV-infected cells via FasL, cytolytic granule secretion (perforin/granzyme), and TRAIL-mediated mechanisms (7, 17, 28). Despite the loss of one of these three pathways, Trail−/− mice were still able to control the infection and clear virus similarly to WT mice after a clinically significant IAV infection. In fact, IAV-specific CD8 T cells from WT and Trail−/− mice exhibited similar in vitro killing capacity, suggesting equivalent cytotoxic ability on a per-cell basis. Examination of the in vivo cytotoxicity mediated by IAV-specific CD8 T cells, in contrast, revealed enhanced target cell killing in clinical dose-infected Trail−/− mice. Notably, these results are in opposition to those observed during the response to a subclinical IAV infection (17). Further, the increased T cell cytotoxicity observed in the Trail−/− mice did not result from a compensatory increase in FasL expression or granzyme B production, but instead correlated with more IAV-specific T cells in the lungs because of increased pulmonary recruitment, decreased apoptosis, and increased proliferation. These data suggest quite different functions for TRAIL during the immune response to IAV depending on the initial infection conditions. Thus, understanding why TRAIL functions differently based on the IAV dose is a challenge for future studies.

One possibility may be that there are TRAIL-dependent differences in dendritic cell function in the lung-draining LN, as well as in the lung environment itself, that contribute to the differential T cell responses observed in the WT and Trail−/− mice after IAV infection—including differences in MHC and costimulatory molecule expression, cytokine/cytokine production, ability to prime naive IAV-specific T cells in the lung-draining LN, and the ability to stimulate T cell survival in the lung (36, 41, 42). Some of these differences in dendritic cell function may be related to the way IAV-derived pathogen-associated molecular patterns and proinflammatory damage-associated molecular patterns (43) released from dying IAV-infected respiratory epithelial cells are perceived by TLR and NLR expressed by different phagocytes in the lung at the time of infection. Diehl et al. (44) reported that Trail−/−Drs3 interactions contributed the negative regulation of proinflammatory cytokine production by dendritic cells and macrophages stimulated with various TLR agonists. Dendritic cells and macrophages can also upregulate TRAIL expression after cytokine or TLR agonist stimulation (44–47). Given the strong evidence for TRAIL as an inducer of apoptosis and the emerging evidence for nonapoptotic TRAIL signaling (48), it is tempting to speculate that TRAIL may serve as a negative regulator of chemokine/cytokine production in these APCs/phagocytes during a clinically significant IAV infection through direct apoptotic and nonapoptotic signals. For example, TRAIL expression could result in the killing of the cells via the canonical apoptotic signaling pathway. Alternatively, TRAIL expression could induce signaling pathways that shut down chemokine production without inducing death. Considering the data suggesting that cIAP is required for inflammasome activation (49), that TRAIL signaling can downregulate cIAP (49), and that IAV activates the inflammasome through NLRP3 (50, 51), the potential intersection of the TRAIL receptor and inflammasome signaling pathways provides an intriguing possibility for how TRAIL might affect this aspect of the immune response during a clinically significant IAV infection.

The experimental system we chose for this investigation used a clinically significant IAV infection to better model the clinical symptoms observed in IAV-infected humans. In particular, this clinically significant IAV infection model increased morbidity and mortality in WT mice, symptoms that were not observed in WT mice given a subclinical infection with the same virus strain (17). One complicating factor in understanding the immune response to primary IAV infection is the highly variable nature of the virus itself. Even among the commonly used laboratory IAV strains, immune responses vary in their dependence/independence on/from regulation by other cell types. This challenge is broadened when one considers highly pathogenic strains like the 1918 strain or the recently emerged H5N1 avian influenza strains. An important determinant of the extent of lung injury in the context of IAV clearance is the relationship between viral load and the magnitude of the IAV-specific CD8 T cell response. Significant lung injury, which can be mediated by the viral infection itself as well as the host immune response, commonly occurs during clinical and experimental IAV infection (52). However, protective immunity and clearance of pulmonary pathogens (including IAV) is tied to cellular immunity (5, 33, 53), particularly the activity of pathogen-specific CD8 T cells. CD8 T cells potently block IAV replication and are protective against illness and IAV-induced lung injury when viral loads are low, but the CD8 T cell response to a high IAV load can result in significant immunopathology (37). Our data suggest that during a clinically significant IAV infection, TRAIL signals have the greatest impact on controlling the magnitude of the IAV-specific CD8 T cell response in the lungs by blunting IAV-specific CD8 T cell recruitment to the lungs, as well as reducing the viability/proliferative capacity of these cells. We have concentrated our experiments and discussion on the influence of TRAIL on the IAV-specific T cell response during a clinically significant infection; however, it is important to note that other immune cell populations can contribute to the immunopathology seen during IAV infection. In particular, TRAIL-expressing macrophages contribute to IAV-induced pneumonia by inducing apoptosis of airway epithelial cells after a lethal infection (30). Data of interest from this study showed that anti-TRAIL treatment attenuated lung epithelial apoptosis, lung leakage, and enhanced survival p.i., suggesting TRAIL directly contributed to the immunopathology (instead of the increased immunopathology seen in our system in the absence of TRAIL). Regardless of these differences, a better understanding of the positive (i.e., clearing virus efficiently and limiting virus-induced pathology) and negative (i.e., immunopathology) roles for TRAIL in the immune response to IAV might aid in improving intervention strategies for the treatment of IAV infections and the symptoms associated with them.

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Disclosures
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Supplemental Figure 1. *Trail*−/− BALB/c mice show enhanced mortality after clinical dose IAV infection compared to WT BALB/c mice. WT or *Trail*−/− BALB/c mice were infected with 1500 EIU A/PR/8/34. Mice were monitored daily for mortality.
Supplemental Figure 2. Trail-/- BALB/c mice show enhanced pulmonary recruitment of antigen-specific T cells after IAV infection. WT or Trail-/- BALB/c mice were infected with 1500 EIU A/PR/8/34. At d 8 p.i., lungs were harvested and homogenized; isolated cells were stained with anti-CD3ε, -CD8α, HA_{529} tetramer, and NP_{147} tetramer. The number of CD8^{+} tetramer^{+} T cells (mean ± SD) from WT or Trail-/- BALB/c mice were enumerated using total pulmonary cell counts and flow cytometry. Bars represent 4-5 mice per group and data are representative of two independent experiments.
Supplemental Figure 3. Trail−/− B6 mice show enhanced pulmonary recruitment of antigen-specific T cells after low virulent IAV infection. WT or Trail−/− B6 mice were infected with 1500 EIU X-31. On d 8 p.i., lungs were harvested, homogenized, and the isolated cells were restimulated in vitro with NP366 or PA224 peptide for 8 h. The cells were then stained with anti-CD3, -CD8α, and –IFNγ mAb. The number of Ag-specific T cells, based on IFNγ production (mean ± SD), from WT or Trail−/− B6 mice were enumerated using total pulmonary cell counts and flow cytometry. Bars represent 4-5 mice per group and data are representative of two independent experiments.
Supplemental Figure 4. Experimental design to measure chemokine-mediated recruitment of IAV-specific CD8 T cells into the lungs of IAV-infected WT and Trail−/− B6 mice. WT or CXCR3−/− donor B6 mice and WT or Trail−/− recipient B6 mice were infected with 1500 EIU A/PR/8/34. On d 6 p.i., lung-draining LN were harvested from the donor mice and homogenized. From these cells, CD8+ cells were MACS purified. The purified populations were then blocked with anti-CCR5 (or isotype) and stained with PKH-26 or PKH-67, as described in the table insert. The blocked/knockout cells were then mixed with an unblocked, C57BL/6 reference cells and 3.5 x 10^5 cells were transferred to recipient mice. After 20 h, the lungs were harvested from the recipient mice, and T cell trafficking was assessed by comparing migration of the blocked/knockout populations to the cotransferred reference population.