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Soluble, but not transmembrane, TNF- α is required during influenza infection to limit the magnitude of immune responses and the extent of immunopathology

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Abstract

TNF- α is a pleiotropic cytokine, which has both proinflammatory and anti-inflammatory functions during influenza infection. TNF- α is first expressed as a transmembrane (mem) protein that is proteolytically processed to release a soluble (sol) form. memTNF- α and solTNF- α have been shown to exert distinct tissue-protective or -pathologic effects in several disease models. However, the relative contributions of memTNF- α or solTNF- α in regulating pulmonary immunopathology following influenza infection are unclear. Therefore, we performed intranasal influenza infection in mice exclusively expressing non-cleavable memTNF- α or lacking TNF- α entirely and examined the outcomes. We found that solTNF- α , but not memTNF- α , was required to limit the size of the immune response and the extent of injury. In the absence of solTNF- α , there was a significant increase in the CD8⁺ T-cell response, including virus-specific CD8⁺ T-cells, which was due in part to an increased resistance to activation-induced cell death. We found that solTNF- α mediates these immunoregulatory effects primarily through TNF receptor 1 (TNFR1), since mice deficient in TNFR1, but not TNFR2, exhibited dysregulated immune responses and exacerbated injury similar to that observed in mice lacking solTNF- α . We also found that solTNF- α expression was required early during infection to regulate the magnitude of the CD8⁺ T-cell response indicating that early inflammatory events are critical for the regulation of the effector phase. Taken together, these findings suggest that processing of memTNF- α to release solTNF- α is a critical event regulating the immune response during influenza infection.

Keywords

TNF- α ; TNF- α processing; influenza; immunopathology; CD8⁺ T cells

Introduction

Influenza A virus is a respiratory pathogen that is capable of causing significant pulmonary pathology in humans (1). Infection of alveolar macrophages and respiratory epithelial cells with virus results in production of cytokines and chemokines such as TNF- α , IL-6, CCL2, CXCL10, and many others (2, 3). This results in progressive recruitment of macrophages, neutrophils, and CD8⁺ T cells into the lungs and airways and virus is cleared 7 to 10 days post-infection (4, 5). CD8⁺ T cells, operating both through direct cytolysis of infected cells and production of cytokines (such as TNF- α and IFN- γ), have a vital role in the clearance of influenza virus from the lung as mice deficient in T cells succumb very late to infection with delayed and muted pulmonary pathology and very high systemic viral titers (6, 7). There are many important variables that can determine the severity of illness and lung injury following influenza infection and it is believed that a dysregulated host response can contribute to a significant portion of this pathology (8, 9).

Traditionally, TNF- α has been considered to be a pro-inflammatory cytokine as it plays an important role mediating many disease processes including sepsis, sarcoidosis, and rheumatoid arthritis, among others (10–12). In this regard, TNF- α has been shown to exacerbate inflammation and enhance morbidity during influenza infection. Neutralization of TNF- α during influenza infection can reduce pulmonary infiltration and lung injury and prolong survival without impairing viral clearance (13, 14). Furthermore, we have shown that in a transgenic mouse model of severe influenza infection, effector CD8⁺ T-cell production of TNF- α is required to induce pulmonary infiltration and diffuse alveolar damage (15, 16). TNF- α mediates its effects by signaling through two distinct receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (17). Consistent with a pro-inflammatory role for TNF- α during influenza infection, mice deficient in TNFR1 exhibit prolonged survival, reduced morbidity, and pulmonary infiltration (18, 19). Furthermore, we have demonstrated that both TNFR1 and TNFR2 signaling contribute to immunopathology during effector CD8⁺ T-cell clearance of influenza virus (15, 20).

Despite strong evidence supporting a pro-inflammatory role for TNF- α , it is becoming increasingly clear that TNF- α is also capable of exerting immunoregulatory effects during infection and disease. The clinical use of anti-TNF- α biological agents such as etanercept, infliximab, adalimumab has been associated with serious adverse events such as lupus and demyelinating disease and an increased risk for infection and certain malignancies (21). In mice, TNF- α deficiency has been shown to exacerbate experimental autoimmune encephalitis and lupus indicating that TNF- α can suppress inflammatory responses (22, 23). It was also recently revealed that TNF- α has an immunosuppressive role during influenza infection (24). In the complete absence of TNF- α , infected mice exhibited increased morbidity and an increased and prolonged CD8⁺ T-cell response, with enhanced inflammation and injury (24). Studies involving mice deficient in IL-15 have supported the hypothesis that the magnitude of the CD8⁺ T-cell response is associated with the extent of lung injury during influenza infection (25). However, the cellular mechanisms by which TNF- α limits the magnitude and duration of the CD8⁺ T-cell response, and mitigation of immunopathology during influenza infection are unclear.

Previous studies investigating the immunoregulatory roles of TNF- α have utilized genetic deletions or antibody neutralization of TNF- α . However, these methods block the effects of both memTNF- α and solTNF- α signaling. TNF- α is first expressed as a transmembrane protein that is proteolytically processed by ADAM17 (a disintegrin and metalloprotease or TNF- α converting enzyme, TACE) to release a solTNF- α (26). Evidence to date indicates that memTNF- α and solTNF- α have both distinct as well as overlapping biological functions. In mice exclusively expressing non-cleavable memTNF- α , it was demonstrated that memTNF- α was sufficient to provide protection against *M. tuberculosis* infection (27). In addition, these mice were also protected against septic shock and pulmonary fibrosis suggesting that memTNF- α did not mediate some of the deleterious effects of solTNF- α while still preserving some protective effects (28, 29). However, the respective roles of memTNF- α and solTNF- α in regulating immune responses and immunopathology during influenza infection remain unclear.

In this study, we investigated the differential impact of memTNF- α and solTNF- α in regulating immune responses to a sub-lethal influenza virus infection using mice that exclusively express a non-cleavable memTNF- α or which were entirely deficient in TNF- α . We found that solTNF- α but not memTNF- α was required early during infection to limit the magnitude of the immune response and the extent of lung immunopathology. In the absence of solTNF- α , there was a significant increase in CD8⁺ T-cell accumulation late in infection, including virus-specific CD8⁺ effector T-cells. The enhanced CD8⁺ T-cell response in the absence of solTNF- α appeared to drive the increased lung injury as depletion of CD8⁺ T cells attenuated the extent of lung injury. Overall, the findings of this study suggest that proteolytic processing of memTNF- α to solTNF- α is a critical immunoregulatory event during influenza infection. Moreover, our observations are important for understanding how early events during infection can shape the contraction of the effector phase and the extent of pathologic injury.

Methods

Mice

Seven-week-old C57BL/6, Thy1.1, and CD45.1 mice or mice deficient in either TNFR1 (TNFR1^{-/-}) or TNFR2 (TNFR2^{-/-}) were purchased from Jackson Laboratories (Bar Harbor, ME). TNF- α knockout (TNF^{-/-}) breeding pair on a C57BL/6 background was purchased from Taconic (Germantown, NY). Breeding pairs of mice that only express membrane-bound TNF- α created by knocking-in a non-cleavable 1–9, K11E TNF- α allele (memTNF^{1–9, K11E} KI) were generously provided by Dr. William Rigby (Dartmouth College) (28). Mice were bred and maintained in a pathogen-free environment and all experiments used seven- to twelve-week-old female mice. All animal studies were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at Geisel School of Medicine at Dartmouth.

Reagents

The following monoclonal antibodies were purchased from Biolegend (San Diego, CA) as conjugated to FITC, PE, PE-Cy7, PerCP-Cy5.5, Alexa-647, or APC-Cy7: CD4 (GK1.5),

CD8 (53-6.7), CD45.1 (A20), CD90.1 (OX-7), CD107a (1D4B), annexin V, and Bcl-2 (BCL/10C4). PE-conjugated tetramer PA₂₂₄₋₂₃₃ and APC-conjugated tetramer NP₃₆₆₋₃₇₄ were prepared by the NIH Tetramer Core Facility (Atlanta, GA). Anti-mouse CD16/32 was purchased from DartLab (Lebanon, NH). For antibody neutralization experiments, anti-TNF- α (XT3.11), anti-CD8a (2.43), and rat IgG1 isotype controls (HRPN, LTF-2) were purchased from BioXCell (West Lebanon, NH). Recombinant mouse solTNF- α was purchased from BioLegend.

Viral infection and treatment

Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine and inoculated intranasally with one-tenth the median lethal dose of mouse-adapted influenza A/PR/8/34 (H1N1) virus. Morbidity as measured by weight loss was monitored daily after infection. Peripheral oxygen saturation (SpO₂) of conscious mice was measured before and after infection using a MouseOx system (Starr Life Sciences Corp., Allison Park, PA). For TNF- α neutralization, mice received 500 μ g of anti-TNF- α or isotype control antibodies by intraperitoneal injection on the days indicated. For CD8 depletion, mice received 300 μ g of anti-CD8a or isotype control antibodies by intraperitoneal injection on days 1 and 4 post-infection. For solTNF- α treatment, 2 μ g of recombinant mouse solTNF- α was intranasally administered at the time of infection.

Viral titers

At 3, 8 and 14 days post-infection, whole lungs were homogenized in PBS, snap frozen, and stored at -80°C. Tenfold serial dilutions of lung samples were applied in triplicate to Madin-Darby canine kidney (MDCK) cells in a 96-well plate and incubated at 37°C for 5 days. Microwells containing influenza virus were identified by chicken red blood cell hemagglutination and the 50% tissue culture infective dose (TCID₅₀) was calculated (30).

Bronchoalveolar lavage (BAL) and tissue preparation

Airway cells and cytokines were collected by lavaging the lungs four times with a single 1 mL volume of PBS via an incision in the trachea. BAL fluid (BALF) was centrifuged and the fluid was stored at -80°C for cytokine and chemokine analysis. ELISA and Millipore Mouse 32-plex Luminex assay was used to determine the expression of cytokines and chemokines. ELISA was used to determine the level of albumin, a marker of vascular leakage, in BALF (Bethyl Laboratories). Lung and mediastinal lymph node (MLN) single-cell suspensions were prepared by passing these tissues through 70 μ m and 40 μ m nylon cell strainers, respectively. Red blood cells were lysed using Gey's solution. Total viable cell counts were obtained by counting the cells on a hemocytometer with trypan blue exclusion. Identification of specific cell populations was determined by analyzing the samples on a flow cytometer as previously described (30). For lung histology, lungs were inflated with 0.5% low melting point agarose in PBS, fixed in formalin, sectioned, and stained with hematoxylin and eosin as previously described (16).

Activation-induced cell death assays

CD8⁺ T cells recovered from BAL on day 8 post-infection were resuspended in complete media with IL-2 and stimulated with 10 µg/mL plate-bound anti-CD3 in a 96-well plate at 37°C, 5% CO₂. After 72 hours, cells were stained with annexin V and the frequency of apoptotic cells was determined by flow cytometry. Bcl-2 expression was analyzed on fixed permeabilized cells by flow cytometry. Alternatively, splenocytes were harvested from day 10 post-infected CD45.1 and TNFR1^{-/-} mice and equal numbers of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells from CD45.1 (CD45.1⁺/Thy1.1⁻) and TNFR1^{-/-} (CD45.1⁻/Thy1.1⁻) mice were adoptively transferred into naive Thy1.1 (CD45.1⁻/Thy1.1⁺) mice one day prior to infection. CD8⁺ T cells were recovered from the BAL on day 7 post-infection and the frequency of transferred NP₃₆₆₋₃₇₄-specific CD8⁺ T cells from CD45.1 and TNFR1^{-/-} mice was determined by flow cytometry.

Flow cytometric analysis

Cells were blocked with anti-mouse CD16/32 and then stained with specific antibodies or isotype controls as described previously (31). For tetramer staining, cells were blocked with anti-mouse CD16/32, incubated with tetramer for 1 hour at room temperature, and stained for additional surface markers. Flow cytometry was performed on a FACS Calibur, FACS Canto cytometer (BD Biosciences, San Jose, CA), or MacsQuant Analyzer (Miltenyi Biotec, San Diego, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA) using one-way ANOVA with Tukey post-hoc tests and 95% confidence interval or two-tailed unpaired t-test with 95% confidence interval. Data are presented as the mean ± standard deviation.

Results

solTNF-α serves to mitigate lung injury during influenza infection

It was recently demonstrated that in the complete absence of TNF-α there is an increase in immunopathology during influenza virus infection, suggesting a dominant anti-inflammatory effect (24). Since memTNF-α and solTNF-α have been shown to have both distinct and overlapping effects, we sought to characterize the roles of memTNF-α and solTNF-α in regulating immunopathology during influenza infection. Therefore, we infected TNF^{-/-}, memTNF^{1-9, K11E} KI, and control wild-type C57BL/6 (WT) mice with a sub-lethal dose of influenza A virus. We found that both memTNF^{1-9, K11E} KI and TNF^{-/-} mice experienced greater weight loss morbidity, compared to WT mice (Figure 1A). Histologically, memTNF^{1-9, K11E} KI and TNF^{-/-} mice exhibited similarly increased alveolar airspace and interstitial inflammatory cell infiltration with increased alveolar damage compared to WT mice (Figures 1B, 1C, 1D). This histological observation of enhanced lung injury prompted our measurement of albumin leakage into the alveoli and we found that both infected memTNF^{1-9, K11E} KI and TNF^{-/-} mice had increased levels of albumin in the airways compared to WT, indicating enhanced disruption of the distal

epithelial barrier function (Figure 1E). The physiologic impact of the pulmonary pathology observed in both memTNF^{1-9, K11E} KI and TNF^{-/-} mice was confirmed by a greater reduction in peripheral oxygen saturation after viral infection compared to WT mice (Figure 1F). Taken together, these results indicate that expression of memTNF- α alone is insufficient to limit the extent of lung injury during clearance of influenza infection and suggest that the anti-inflammatory effects of TNF- α are mediated by solTNF- α .

Both solTNF- α and memTNF- α are dispensable for influenza virus clearance

Next, we measured influenza viral titers in whole lung homogenates to investigate the role of memTNF- α and solTNF- α in viral clearance. All mice were capable of complete virus clearance, as no virus was detected in the lungs 14 days post infection (Figure 1G). Importantly, no significant differences in viral titers were observed on days 3 or 8 post-infection (Figure 1G). These data strongly suggest that the enhanced morbidity and pathology we observed in memTNF^{1-9, K11E} KI and TNF^{-/-} mice was not due to inability of the host to control or clear the virus.

Dysregulated cytokine and chemokine responses in the absence of solTNF- α

To investigate the molecular mechanisms by which solTNF- α mitigates lung injury during influenza infection, we examined the expression of cytokines and chemokines in the airways 8 days post-infection. As expected solTNF- α was present in lavage samples from WT mice but not detected in samples recovered from memTNF^{1-9, K11E} KI and TNF^{-/-} mice (Figure 2A). However, total TNF- α production does not appear to be affected in memTNF^{1-9, K11E} KI mice, as TNF- α expression in stimulated NP₃₆₆₋₃₇₄-specific CD8⁺ T cells derived from memTNF^{1-9, K11E} KI mice was comparable to that of WT CD8⁺ T cells (Supplemental Figure 1). We observed increases in the BALF levels of IL-6 and IL-15 in the airways of memTNF^{1-9, K11E} KI and TNF^{-/-} mice compared to WT mice (Figures 2B, 2C). Both of these cytokines have important roles in regulating the magnitude of the T-cell response to influenza infection (25, 32). Moreover, there were increases in the levels of CCL2 and CXCL10 in the airways of memTNF^{1-9, K11E} KI and TNF^{-/-} mice compared to WT mice (Figures 2D, 2E). This is similar to the enhanced levels of CCL2 and CXCL10 observed by Damjanovic *et al.* in the airways of TNF^{-/-} mice during influenza infection (24). We also observed enhanced levels of MIP-1 α , MIP-1 β , and G-CSF in the airways of memTNF^{1-9, K11E} KI and TNF^{-/-} mice compared to WT mice (Figures 2F, 2G, 2H). Following infection with highly pathogenic influenza viruses, higher levels of CCL2, MIP-1 α , MIP-1 β , IL-6, and G-CSF in the lungs and higher levels of CCL2, CXCL10, and IL-6 in the serum have been observed in mice and humans, respectively (9, 33–35). These data suggest that solTNF- α expression functions in part to limit cytokine and chemokine expression following influenza infection.

solTNF- α limits the magnitude of the CD8⁺ T-cell response during influenza infection

Since we observed a dysregulation of cytokine and chemokine expression, we next investigated whether there were differences in the immune response that could contribute to the increased lung injury we observed after infection in memTNF^{1-9, K11E} KI and TNF^{-/-} mice. We recovered cells from the airways, whole lung, and MLN harvested from animals 8

days after infection and found an increase in the total number of cells present in the airways, lung, and MLN of both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice (Figures 3A, 3B, 3C). Using flow cytometry to identify specific cell populations, we observed a significant enhancement in the CD8⁺ T-cell response in all tissues examined of both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice (Figures 3D, 3E, 3F). Total numbers of CD4⁺ T cells were also increased in both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice (Supplemental Figures 2A, 2B, 2C). To characterize further the CD8⁺ T-cell response, we examined the virus-specific response to the H-2k^b-restricted influenza virus CD8⁺ T-cell epitopes, NP₃₆₆₋₃₇₄ and PA₂₂₄₋₂₃₃. We found a greater number of both NP₃₆₆₋₃₇₄-specific and PA₂₂₄₋₂₃₃-specific CD8⁺ T cells in both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice (Figures 3G, 3H, 3I; Supplemental Figures 2D, 2E, 2F). The enhanced influenza-specific CD8⁺ T-cell response that was observed in the absence of TNF- α was due in part to the elevated levels of IL-15 observed in the airways of these mice, as blockade of IL-15 signaling attenuated both the total and NP₃₆₆₋₃₇₄-specific CD8⁺ T-cell responses in TNF^{-/-} mice (Supplemental Figures 3A, 3B). These data indicate that TNF- α serves to limit the magnitude of the influenza-specific T-cell response. We also found a significant increase in the total number of monocyte-derived macrophages and neutrophils in the airways of both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice on day 8 post-infection (data not shown), indicating that solTNF- α expression was also required to limit the infiltration by other inflammatory cell types following influenza viral challenge.

Enhanced CD8⁺ T-cell responses promote lung injury in the absence of solTNF- α

Since we observed an enhanced CD8⁺ T-cell response in both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice (Figures 3B, 3C, 3D), at a time that corresponded with the peak in morbidity (weight loss; Figure 1A), we hypothesized that the increased magnitude of the CD8⁺ T-cell response and effector function promoted the enhanced injury observed in these mice. We first examined the total number of cytotoxic CD8⁺ T cells expressing CD107a, a marker of recent degranulation, in the airways and lungs following influenza infection. We observed an increase in the total number of cytotoxic CD8⁺ T cells expressing CD107a in the airways and lungs of both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, compared to WT mice on day 8 post-infection suggesting that increased CD8⁺ T-cell cytotoxicity could lead to enhanced lung injury (Figure 3J, 3K). To test whether the increased number of cytotoxic CD8⁺ T cells in the absence of TNF- α contributed to the enhanced lung injury, we depleted CD8⁺ T-cells in TNF^{-/-} mice by administering anti-CD8a antibody on days 1 and 4 post-infection. Following CD8⁺ T-cell depletion, we observed a reduction in weight loss morbidity on day 8 post-infection (Figure 4A). In contrast to TNF^{-/-} mice that received control antibody (Figure 4B), antibody depletion of CD8⁺ T cells reduced infiltration of the airspaces and limited the extent of alveolar damage (Figure 4C). The reduction in immunopathology, which was evident on histologic analysis, corresponded to a reduction in BALF albumin in the airways of CD8⁺ T-cell depleted mice after infection (Figure 4D). Taken together, these data indicate that the enhanced CD8⁺ T-cell response that occurs in the absence of TNF- α drives enhanced lung injury during influenza infection and we infer, based upon the data presented above, that solTNF- α regulates the extent of the CD8⁺ T-cell response.

TNFR1 signaling is required to limit the magnitude of the CD8⁺ T-cell response and the subsequent lung injury

To examine the mechanisms by which solTNF- α constrains the T-cell response during influenza infection, we challenged TNFR1^{-/-} or TNFR2^{-/-} mice with influenza virus and examined the inflammatory responses. We found that TNFR1^{-/-}, but not TNFR2^{-/-}, mice recapitulated many of the effects we observed in both memTNF^{1-9, K11E} KI and TNF^{-/-} mice. TNFR1^{-/-} mice exhibited enhanced morbidity, as indicated by a greater loss in percent baseline weight compared to WT mice on day 8 post-infection (Figure 5A). TNFR1^{-/-} mice also exhibited more severe lung injury as measured by elevated albumin levels in the airway compared to WT mice (Figure 5B). Interestingly, TNFR2^{-/-} mice experienced less weight loss and lung injury when compared to WT mice (Figures 5A, 5B). We also observed dysregulated cytokine and chemokine responses in TNFR1^{-/-} mice compared to WT mice (Figure 6). Similar to memTNF^{1-9, K11E} KI and TNF^{-/-} mice, there were elevated levels of IL-6, IL-15, CCL2, CXCL10, MIP-1 α , MIP-1 β , and G-CSF in the airways of TNFR1^{-/-} mice when compared to WT mice (Figure 6). Interestingly, solTNF- α was elevated in the airways of both TNFR1^{-/-} and TNFR2^{-/-} compared to WT mice suggesting that these receptors may play an important feedback role in regulating the levels of solTNF- α in the lung (Figure 6A). Consistent with the exacerbated injury and inflammation, there was an increase in the total number of inflammatory cells recovered from the BAL of TNFR1^{-/-} mice compared to WT mice (Figure 5C). We also observed an increase in the total number of monocyte-derived macrophages and neutrophils in the BAL of TNFR1^{-/-} mice compared to WT mice (data not shown). In contrast, no differences in the total number of cells and total number of monocyte-derived macrophages or neutrophils were observed between WT and TNFR2^{-/-} mice (Figure 5C, data not shown). Moreover, the total number, NP₃₆₆₋₃₇₄-specific, and cytotoxic CD107a expressing CD8⁺ T cells were increased in the airways of TNFR1^{-/-} mice compared to WT mice 8 days after influenza infection (Figures 5D, 5E, 5F). Despite having intact solTNF- α and memTNF- α , TNFR1^{-/-} mice exhibited enhanced CD8⁺ T-cell responses and exacerbated pulmonary pathology following influenza infection. This is consistent with the general belief that solTNF- α primarily mediates its effects through TNFR1 (36, 37) and reinforces our hypothesis that solTNF- α signaling primarily through TNFR1 is required to limit the magnitude of the effector CD8⁺ T-cell response and the extent of lung immunopathology during influenza infection. The attenuated lung injury and reduced cytotoxic CD8⁺ T-cell response in TNFR2^{-/-} mice compared to WT mice (Figures 5D, 5E, 5F) further suggests that the magnitude of the CD8⁺ T-cell response likely impacts the extent of immunopathology following influenza infection.

CD8⁺ T cells primed in the absence of solTNF- α are more resistant to activation-induced cell death

Since we observed an increased CD8⁺ T-cell response in the absence of solTNF- α , we hypothesized that the enhanced magnitude of the CD8⁺ T-cell response could be due to either reduced cell death or enhanced proliferation. We assessed these possibilities and we did not observe any differences in the ability of CD8⁺ T cells recovered from WT, memTNF^{1-9, K11E} KI, or TNF^{-/-} mice to proliferate *ex vivo* in response to anti-CD3

stimulation (data not shown). However, as shown in Figure 7A, we found that CD8⁺ T cells recovered from both memTNF^{1-9, K11E} KI and TNF^{-/-} mice were more resistant to activation-induced cell death than CD8⁺ T cells from WT mice, as indicated by a lower frequency of annexin V⁺ in both memTNF^{1-9, K11E} KI and TNF^{-/-} CD8⁺ T cells, compared to WT CD8⁺ T cells after 72 hours of restimulation with anti-CD3. The increased resistance to activation-induced cell death in both memTNF^{1-9, K11E} KI and TNF^{-/-} CD8⁺ T cells correlated with enhanced expression of the anti-apoptotic protein, Bcl-2, compared to WT CD8⁺ T cells (Figure 7B). To further test this hypothesis, we performed a dual-transfer experiment where we transferred equal numbers of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells from WT and TNFR1^{-/-} mice into a congenic recipient that was subsequently infected with influenza virus. When we harvested cells from the airways 7 days post-infection, we recovered a significantly greater frequency of TNFR1^{-/-} NP₃₆₆₋₃₇₄-specific CD8⁺ T cells compared to transferred WT cells (Figure 7C). Taken together, these data suggest that solTNF- α affects the susceptibility of CD8⁺ T cells to activation-induced cell death and that the increased resistance of CD8⁺ T cells to cell death observed in the absence of solTNF- α may contribute to the enhanced CD8⁺ T-cell responses observed following influenza infection.

solTNF- α has a predominant effect early during infection to limit the magnitude of the T-cell response

solTNF- α has been shown to sensitize T cells to activation-induced cell death during T-cell priming but not restimulation *in vitro* (38). Since we observed resistance to activation-induced cell death in effector CD8⁺ T cells derived from memTNF^{1-9, K11E} KI and TNF^{-/-} mice, we examined whether solTNF- α was required during T-cell priming *in vivo* to limit the magnitude of the T-cell response during influenza infection. To test this, we used a TNF- α neutralizing antibody to deplete TNF- α at different time points during influenza infection in WT mice. We injected anti-TNF- α (or IgG1 control antibody) during T-cell priming (one day prior to infection and days 1 and 3 post-infection) or during the effector phase (days 5 and 7 post-infection). We found that TNF- α depletion early during T-cell priming, but not later during the effector phase, resulted in an increase in the total number of cells recovered from the airways 8 days post-infection compared to IgG1 control treated mice (Figure 8A). Moreover, there was an increase in the total number of CD8⁺ T cells in the airway following early TNF- α depletion compared to later TNF- α depletion or IgG1 control (Figure 8B). The enhancement in the CD8⁺ T-cell response included the virus-specific response, as there were greater numbers of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells following early TNF- α depletion (Figure 8C). To further test whether TNF- α has a predominant effect early during infection, we intranasally administered mouse recombinant solTNF- α to memTNF^{1-9, K11E} KI mice at the time of infection. We found that solTNF- α delivery reduced the total number of cells recovered from the airways on day 8 post-infection, which included a reduction in the total number and NP₃₆₆₋₃₇₄-specific CD8⁺ T-cell responses (Figures 8D, 8E, 8F). These data indicate that early inflammatory events can shape the effector response. Moreover, it suggests that TNF- α expression (and by inference solTNF- α), as well as TNFR1, early after infection is critical for regulating the magnitude of the T-cell response during the effector phase of immune-mediated clearance of influenza infection.

Discussion

In this study, we demonstrated that expression of solTNF- α was critical in regulating immunopathology during influenza infection. Neither memTNF- α nor solTNF- α were required for clearance of influenza virus from the lungs. However, solTNF- α expression was required to limit pulmonary inflammation and infiltration. Increased cytokine and chemokine expression as well as increased cellular infiltration, including an increase in the virus-specific CD8⁺ T-cell response, was observed in memTNF^{1-9, K11E} KI and TNF^{-/-} mice following a sub-lethal influenza infection. This enhanced CD8⁺ T-cell response in the absence of solTNF- α appeared to drive exacerbated lung injury as depletion of CD8⁺ T cells in TNF^{-/-} mice attenuated the extent of pulmonary pathology. The immunoregulatory effects of solTNF- α appear to be mediated by TNFR1, since TNFR1^{-/-}, but not TNFR2^{-/-}, mice recapitulated the enhanced immune responses and exacerbated injury observed in infected memTNF^{1-9, K11E} KI and TNF^{-/-} mice. Furthermore, we found that solTNF- α expression in the first few days after influenza infection was required to limit the effector CD8⁺ T-cell response later during infection, suggesting that proteolytic processing of TNF- α during the priming phase in influenza infection is required to limit the immune response and mitigate lung immunopathology.

The observation that TNF- α was required to limit the size and duration of the immune response and pulmonary injury in the clearance of influenza infection is consistent with recent observations made by Damjanovic *et al.*, who also identified an immunoregulatory role for TNF- α during influenza infection (24). Despite the use of male mice and a different mouse-adapted strain of H1N1 influenza virus, the results were quite similar. Both their study and ours report increased morbidity and pulmonary inflammation (with cellular infiltration) in the absence of TNF- α . Our study identifies solTNF- α as the form of TNF- α required to regulate the immune response and limit lung injury during influenza infection since memTNF^{1-9, K11E} KI mice exhibited a phenotype most similar to TNF^{-/-} mice. We also identified TNFR1 as the TNFR required to mediate the immunoregulatory effects of solTNF- α since TNFR1^{-/-} mice experienced increased morbidity and an enhanced T-cell response. Similarly, both TNF- α and TNFR1 have been implicated in limiting the magnitude and duration of the CD8⁺ T-cell response during lymphocytic choriomeningitis virus infection (39, 40). Interestingly, we found a significant attenuation of injury despite no differences in the total number of cells present in the airways of TNFR2^{-/-} mice when compared to WT mice. However, there were fewer cytotoxic CD8⁺ T cells, including fewer virus-specific CD8⁺ T cells, recovered from the airways of TNFR2^{-/-} mice suggesting that the attenuated injury observed in these mice was due in part to failure in sustaining an antiviral T-cell response. This is consistent with previous studies that reported deficiencies in cytotoxic CD8⁺ T-cell survival and function in mice lacking TNFR2 (41, 42). These observations suggest that solTNF- α signaling through TNFR1 is required to limit immune responses during influenza infection and TNFR2 signaling may be required for survival and cytotoxic function of CD8⁺ T cells.

Our observations suggest that the enhanced morbidity that occurred in the absence of solTNF- α or TNFR1, the peak of which occurred 8 days post-infection during a time when the T-cell response was also peaking, was largely driven by virus-specific CD8⁺ T-cell

cytotoxicity. We observed a significantly greater number of CD8⁺ T cells expressing CD107a, a marker for cells that have recently degranulated, in the airways of memTNF^{1-9, K11E} KI, TNF^{-/-}, and TNFR1^{-/-} mice. Moreover, depletion of CD8⁺ T cells in TNF^{-/-} mice attenuated lung injury after influenza infection. It is not unprecedented for injury to be associated with acquisition of cytotoxic function by CD8⁺ T cells. For example, the onset of hepatocellular injury in a model of hepatitis B virus (HBV) infection, measured by serum alanine aminotransferase levels, was concurrent with the acquisition of cytotoxic activity by HBV-specific CD8⁺ T cells (43). Furthermore, IL-15 has been shown to partly contribute to influenza immunopathology by regulating the virus-specific CD8⁺ T cell response (25). We observed elevated IL-15 levels in the airways of both memTNF^{1-9, K11E} KI and TNF^{-/-} mice, and blockade of IL-15 signaling in TNF^{-/-} mice attenuated CD8⁺ T-cell responses. This suggests that the enhanced IL-15 levels in the absence of solTNF- α may contribute to the increased magnitude of the CD8⁺ T-cell response and the subsequent exacerbation in lung injury. The caveat here is that while increased cytotoxicity may contribute to enhanced injury, it may also be required for clearance of influenza virus from the lungs by CD8⁺ T cells (44). Thus, the CD8⁺ T-cell response needs to be tightly regulated to ensure specific elimination of infected target cells and successful resolution of infection while minimizing both the killing of uninfected bystander cells and release of proinflammatory mediators.

Strikingly, we found that TNF- α expression was required during T-cell priming to limit the size of the effector response. It has been suggested that inflammation early during infection is important in mediating CD8⁺ T-cell contraction (45, 46). Neutralization of TNF- α early during infection but not later during the effector phase resulted in an increase in the number of CD8⁺ T cells, including virus-specific CD8⁺ T cells. Furthermore, we found that CD8⁺ T cells that were primed in memTNF^{1-9, K11E} KI and TNF^{-/-} mice were more resistance to activation-induced cell death. This may have been due in part to increased expression of anti-apoptotic proteins such as Bcl-2. Consistent with these observations, it was previously reported that solTNF- α was required during T-cell priming *in vitro* to sensitize these cells to activation-induced cell death (38). This suggests that effector CD8⁺ T cells generated when TNF- α is neutralized during priming or in a TNF-deficient environment may be intrinsically different from effector CD8⁺ T cells generated in a TNF-sufficient environment. However, we still observed significant contraction of the T-cell response in WT mice and both memTNF^{1-9, K11E} KI and TNF^{-/-} mice. Despite T-cell contraction, there remained a significantly higher number of virus-specific CD8⁺ T cells present in the airways of these mice and raises the possibility that enhanced T-cell numbers may persist to memory and provide better protection against a secondary challenge.

These observations also raise the critical question of whether TNF- α acts directly on T cells during priming to limit the size of the effector phase or if TNF- α acts indirectly to regulate the T-cell response. It was recently shown in an adoptive transfer model that TNFR2-deficient adoptively transferred virus-specific CD8⁺ T cells showed significantly impaired contraction, indicating that TNF- α can act directly on the T-cell to regulate its response (47). It has also been demonstrated that TNF- α may have an important role in the activation and survival of several immunosuppressive cell types. TNF- α signaling has been shown to be

important for the accumulation of myeloid-derived suppressor cells at the site of inflammation and for their suppressive activity (48, 49). Moreover, TNFR1-dependent TNF- α signaling has been implicated in the development of macrophages capable of suppressing T-cell proliferation *in vitro* (50). TNF- α may also be required for the expansion and suppressive function of CD4⁺CD25⁺ regulatory T cells (51). Therefore, TNF- α deficiency may impair the development of regulatory cells, which may contribute to limiting the size of the immune response and minimize injury during influenza infection. Further studies are required to determine whether mice lacking solTNF- α have any changes in the absolute number or function of suppressor cells.

In conclusion, proteolytic processing of TNF- α is a critical event regulating the magnitude and duration of the immune response to influenza infection. Furthermore, solTNF- α expression is required early during infection to limit the magnitude of the effector response. This may have important implications for adverse events following the use of anti-TNF- α biological agents. Whereas neutralization of TNF- α later during infection may reduce inflammation and limit injury, TNF- α neutralization early during infection and disease may actually enhance the inflammatory response and exacerbate immunopathology. The critical sources of TNF- α early during infection that limit the immune responses remain to be determined and future studies are required to elucidate the cellular mechanisms by which TNF- α expression during T-cell priming limits the magnitude of the effector response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

BAL	bronchialveolar lavage
BALF	bronchoalveolar lavage fluid
MLN	mediastinal lymph node
memTNF-α	transmembrane TNF-α

solTNF- α soluble TNF- α

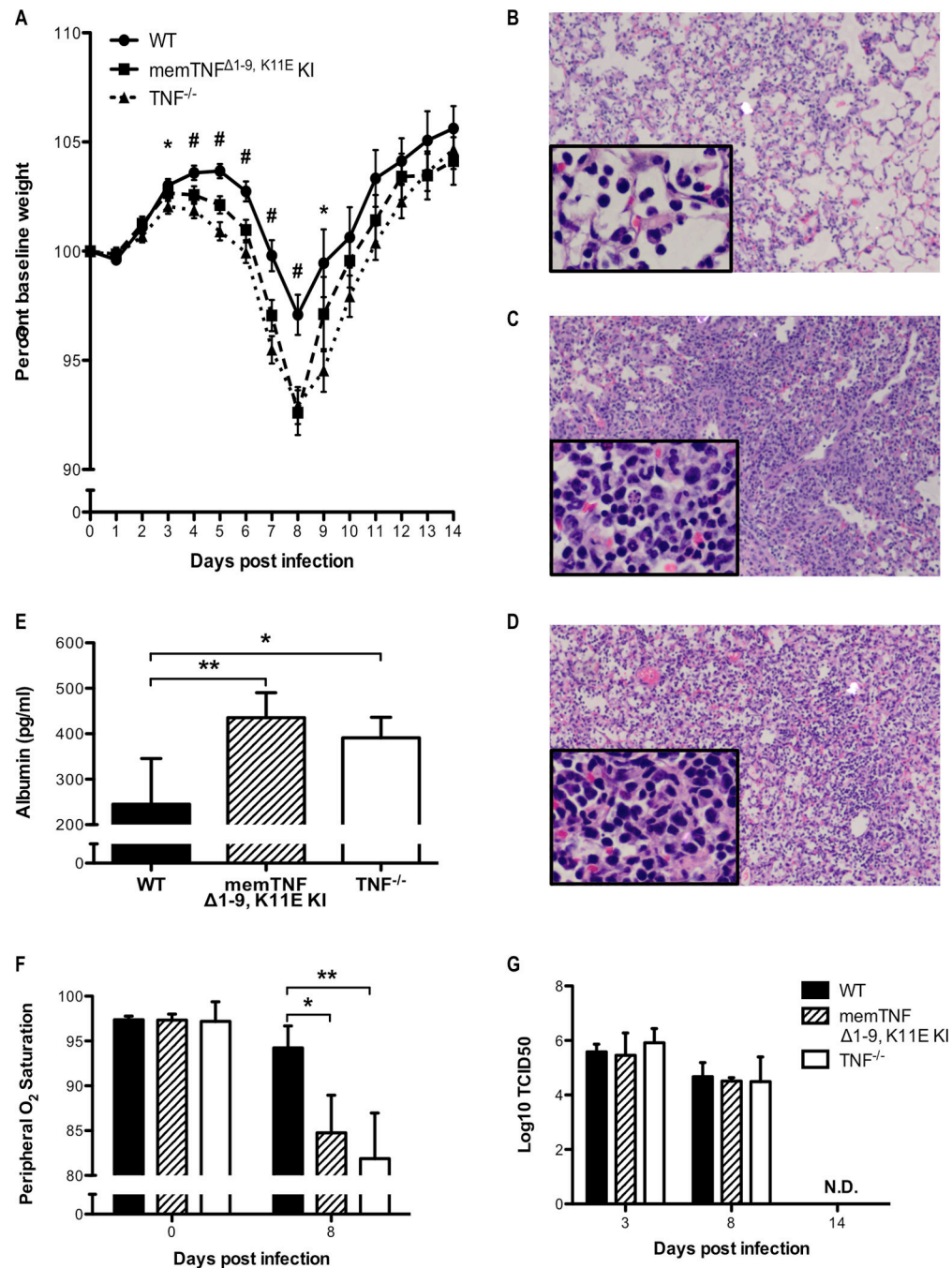


Figure 1. Enhanced morbidity and lung injury in memTNF Δ 1-9, K11E KI and TNF $^{-/-}$ mice following influenza infection

WT, memTNF Δ 1-9, K11E KI, and TNF $^{-/-}$ mice were infected with one-tenth the median lethal dose of mouse-adapted influenza A/PR/8/34 virus. (A) Weight loss was monitored daily after infection and the percent change in baseline weight was calculated. Data represents mean \pm standard error from multiple independent experiments (initial n value is 88 for WT, 75 for memTNF Δ 1-9, K11E KI, and 79 for TNF $^{-/-}$). # P <0.05 memTNF Δ 1-9, K11E KI or TNF $^{-/-}$ compared to WT and * P <0.05 TNF $^{-/-}$ compared to WT. Representative H&E stained lung sections from (B) WT, (C) memTNF Δ 1-9, K11E KI, and (D) TNF $^{-/-}$ mice

harvested 8 days post-infection are shown at 10× magnification with 40× inset. (E) BALF was assessed for albumin by ELISA on day 8 post-infection. (F) Peripheral oxygen saturation levels were measured by the MouseOx System. (G) Viral titers were determined from whole-lung homogenates using the TCID₅₀ method. Data represent mean ± standard deviation. Each group consists of 3–5 mice. Data are representative of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

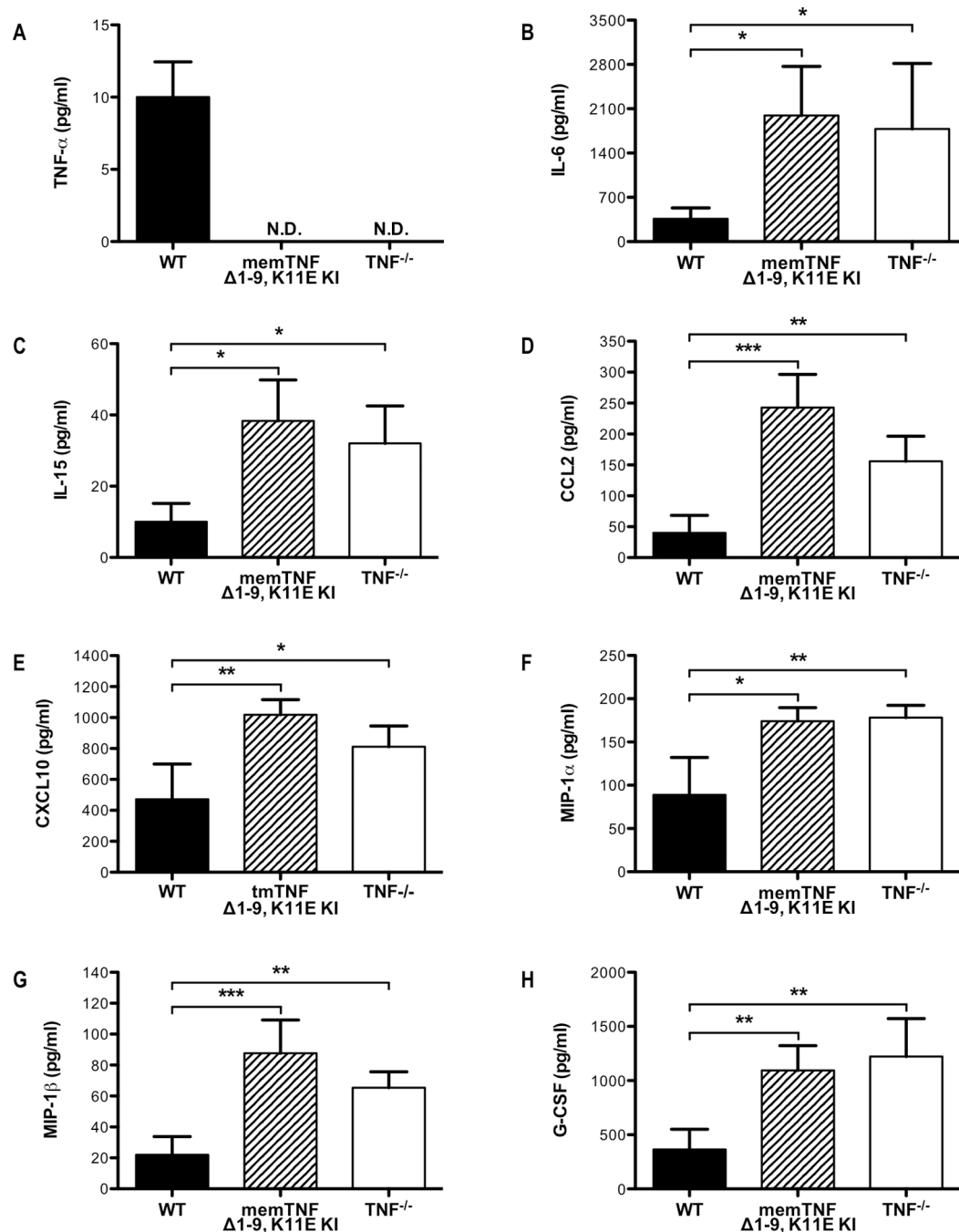


Figure 2. Dysregulated cytokine and chemokine responses in memTNF Δ 1-9, K11E KI and TNF $^{-/-}$ mice following influenza infection

WT, memTNF Δ 1-9, K11E KI, and TNF $^{-/-}$ mice were infected with influenza virus. BALF was recovered on day 8 post-infection and the levels of (A) TNF- α , (B) IL-6, (C) IL-15, (D) CCL2, (E) CXCL10, (F) MIP-1 α , (G) MIP-1 β , and (H) G-CSF were determined by Millipore Mouse 32-plex Luminex assay. Data represent mean \pm standard deviation. Each group consists of 4–6 mice from one or two independent experiments. * P <0.05, ** P <0.01, *** P <0.005.

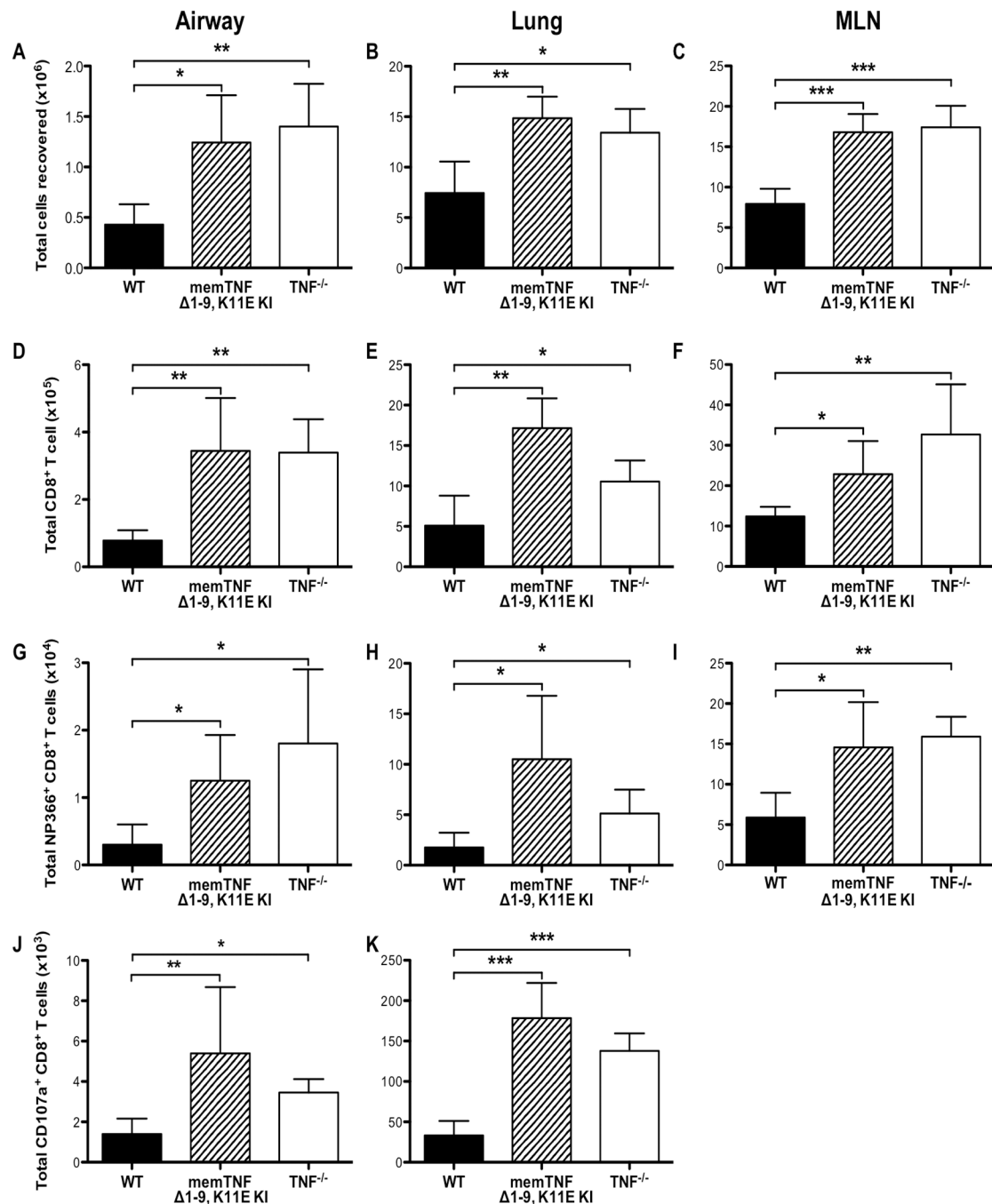


Figure 3. Enhanced CD8 $^{+}$ T-cell responses in memTNF $^{1-9}$, K11E KI and TNF $^{-/-}$ mice following influenza infection

WT, memTNF $^{1-9}$, K11E KI, and TNF $^{-/-}$ mice were infected with a sub-lethal dose of influenza virus and cells were harvested from the airways, lung, and MLN on day 8 post-infection. Total number of viable cells recovered from (A) BAL, (B) lung, and (C) MLN. Total number of CD8 $^{+}$ T cells from (D) BAL, (E) lung, and (F) MLN. Total number of NP₃₆₆₋₃₇₄-specific CD8 $^{+}$ T cells from (G) BAL, (H) lung, and (I) MLN. Total number of CD107a $^{+}$ CD8 $^{+}$ T cells from (J) BAL and (K) lung. Data represent mean \pm standard

deviation. Each group consists of 4–6 mice. Data are representative of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

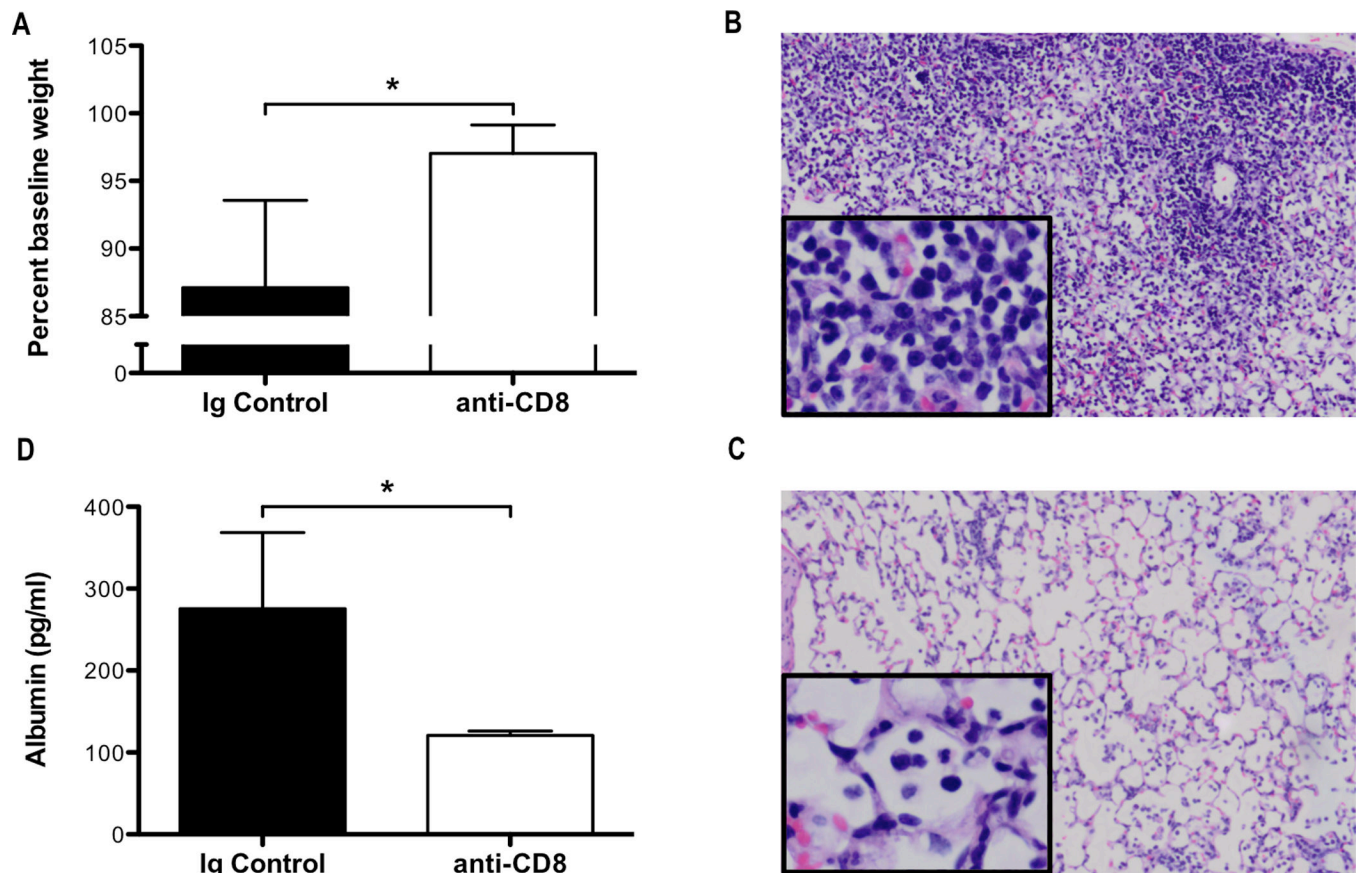


Figure 4. Depletion of CD8⁺ T cells in TNF^{-/-} attenuates lung injury following influenza infection

Influenza infected TNF^{-/-} mice were intraperitoneally administered 300μg of anti-CD8 or rat IgG1 on days 1 and 4 post-infection. (A) Weight loss was monitored daily and the percent baseline weight change for day 8 post-infection was calculated. Representative H&E stained lung sections from mice receiving (B) rat IgG1 or (C) anti-CD8 harvested 8 days post-infection are shown at 10× magnification with 40× inset. (D) ELISA was used to determine the levels of albumin in the BALF 8 days post-infection. Data represent mean ± standard deviation. Each group consists of 3–4 mice. Data are representative of two independent experiments. **P*<0.05.

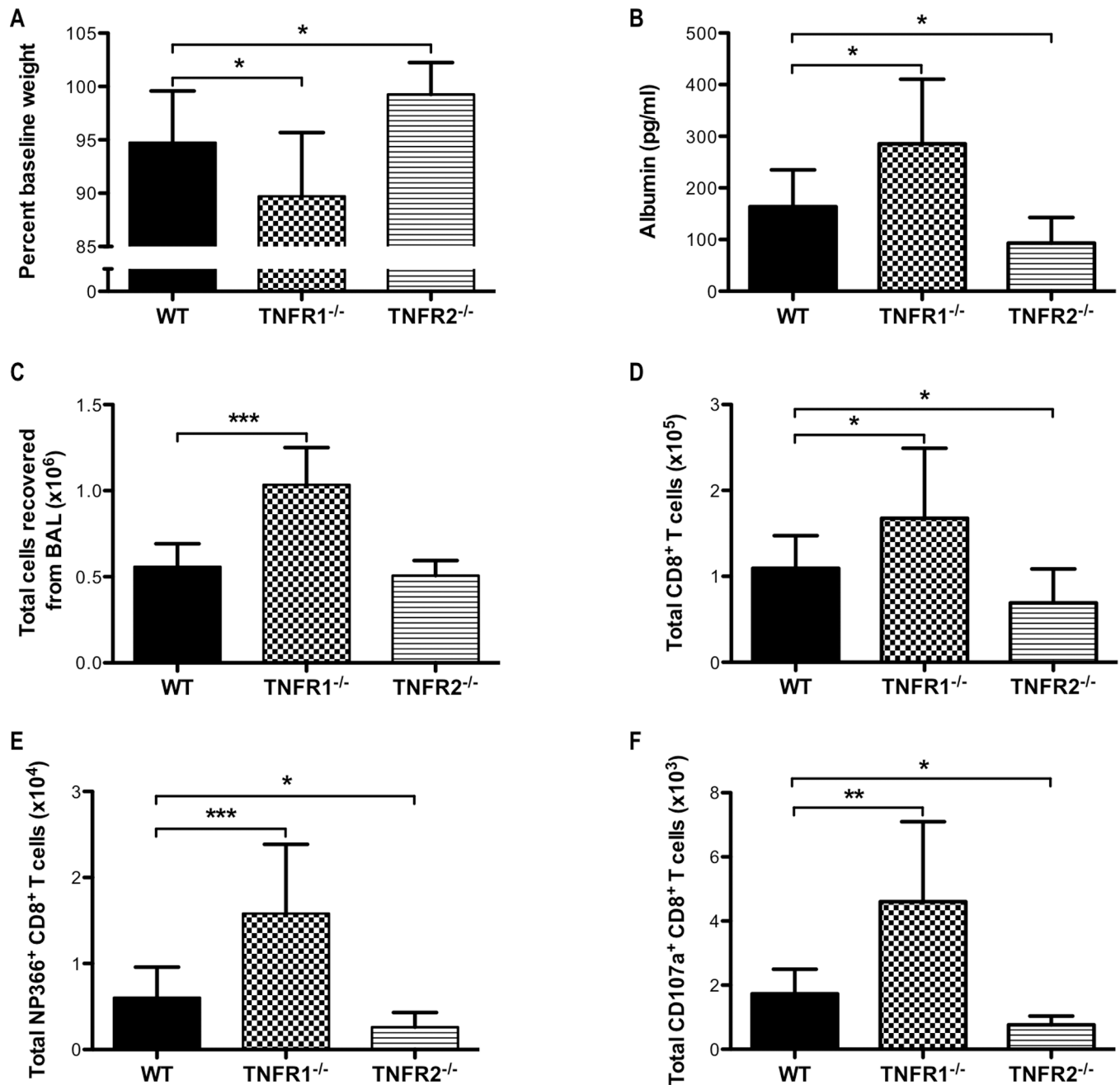


Figure 5. Enhanced lung injury and CD8⁺ T-cell responses in TNFR1^{-/-} mice following influenza infection

WT, TNFR1^{-/-}, and TNFR2^{-/-} mice were infected with a sub-lethal dose of influenza virus. (A) Weight loss was monitored daily and the percent baseline weight change for day 8 post-infection was calculated. (B) ELISA was used to determine the levels of albumin in the BALF 8 days post-infection. (C) The total number of viable cells recovered from BAL day 8 post-infection. Flow cytometry was used to analyze the total number of (D) CD8⁺, (E) NP₃₆₆₋₃₇₄-specific CD8⁺, and (F) CD107a⁺CD8⁺ T cells. Data represent mean \pm standard deviation. Each group consists of 4–6 mice. Data are representative of at least two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

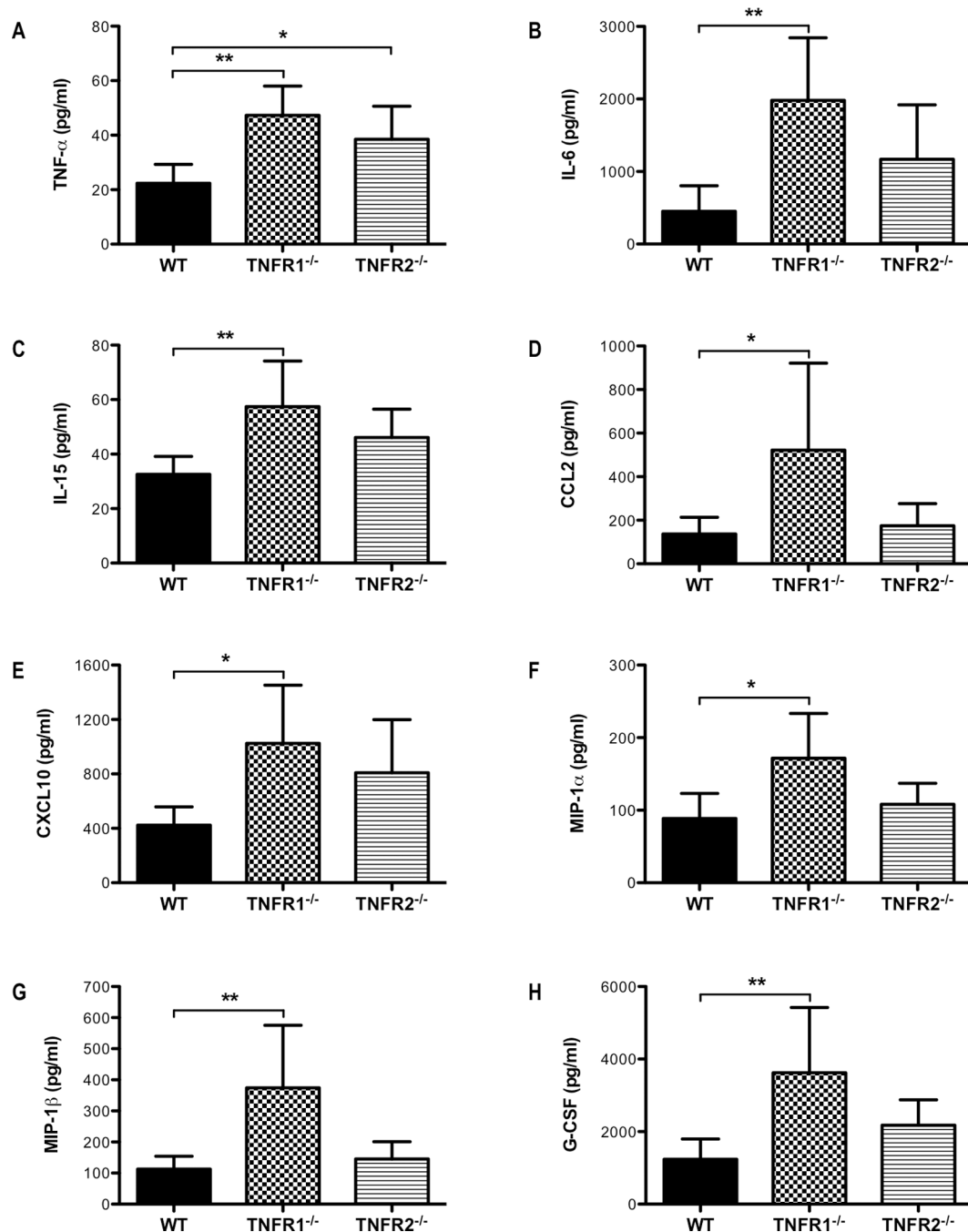


Figure 6. Dysregulated cytokine and chemokine responses in TNFR1^{-/-} mice following influenza infection

WT, TNFR1^{-/-}, and TNFR2^{-/-} mice were infected with influenza virus. BALF was recovered on day 8 post-infection and the levels of (A) TNF-α, (B) IL-6, (C) IL-15, (D) CCL2, (E) CXCL10, (F) MIP-1α, (G) MIP-1β, and (H) G-CSF were determined by Millipore Mouse 32-plex Luminex assay. Data represent mean ± standard deviation. Each group consists of 4–6 mice from one or two independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.005.

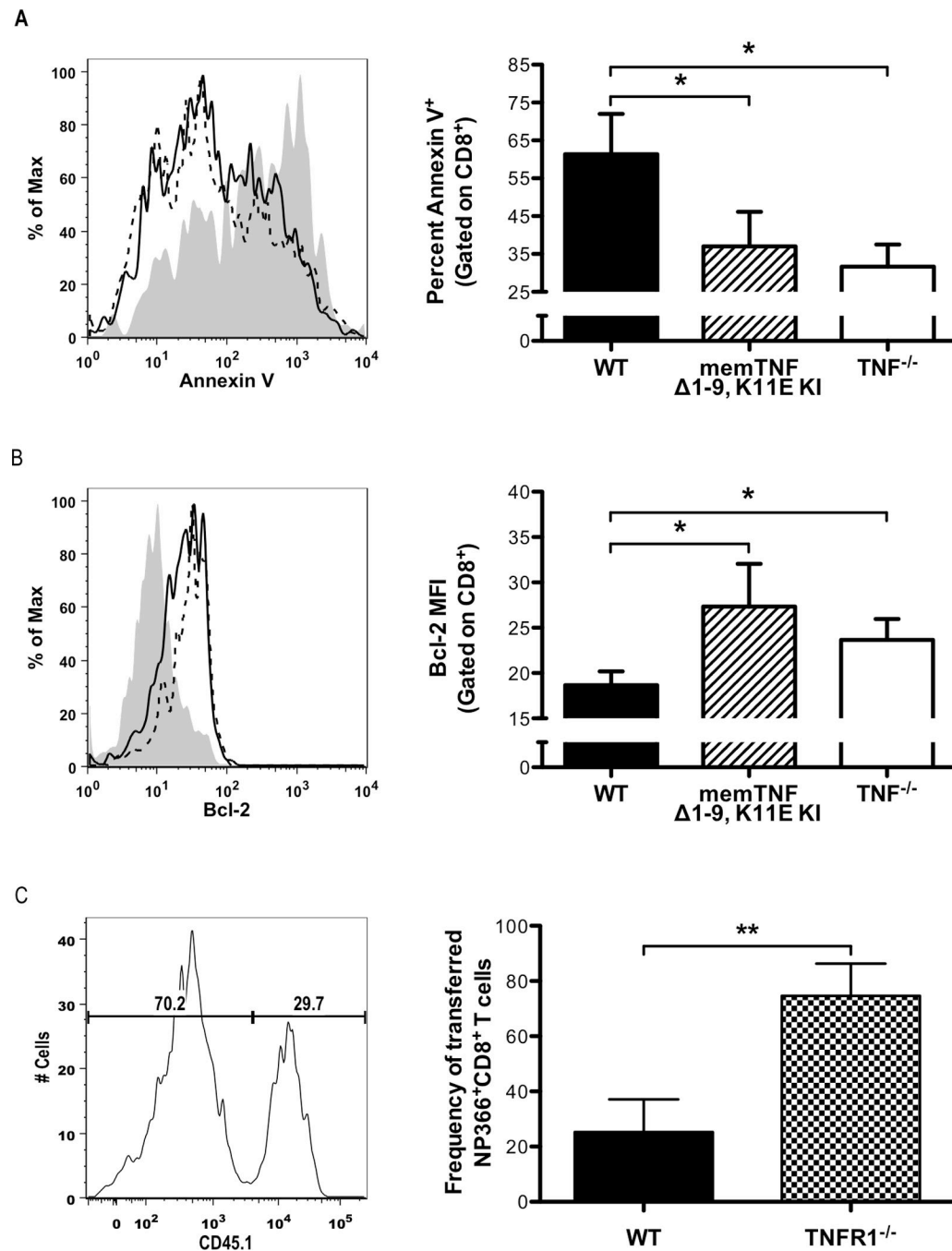


Figure 7. CD8⁺ T cells primed in the absence of solTNF- α are less susceptible to activation-induced cell death

CD8⁺ T cells were recovered from the airways of WT, memTNF^{1-9, K11E} KI, and TNF^{-/-} mice 8 days post-influenza infection. Cells were restimulated *in vitro* for 72 hours with plate-bound anti-CD3. (A) Frequency of annexin V⁺CD8⁺ T cells with representative histogram (left) and quantitative graph (right). Gray represents WT, solid line represents memTNF^{1-9, K11E} KI, and dashed line represents TNF^{-/-}. (B) Bcl-2 expression in CD8⁺ T cells with representative histogram (left) and quantified MFI graph (right). Splenocytes containing equal numbers of NP₃₆₆₋₃₇₄-specific CD8⁺ T cells from day 10 post-infection

WT (CD45.1⁺/Thy1.1⁻) and TNFR1^{-/-} (CD45.1⁻/Thy1.1⁻) mice were adoptively transferred into naive Thy1.1 (CD45.1⁻/Thy1.1⁺) mice one day before infection. (C) On day 7 post-infection, cells were recovered from the lung and the frequency of transferred NP₃₆₆₋₃₇₄-specific CD8⁺ T cells from WT (CD8⁺/NP₃₆₆₋₃₇₄⁺/CD45.1⁺/Thy1.1⁻) and TNFR1^{-/-} (CD8⁺/NP₃₆₆₋₃₇₄⁺/CD45.1⁻/Thy1.1⁻) mice were determined with representative histogram (left) and quantified MFI graph (right). Data represent mean \pm standard deviation. Data are representative of at least two independent experiments with 3 mice per group. * P <0.05, ** P <0.01.

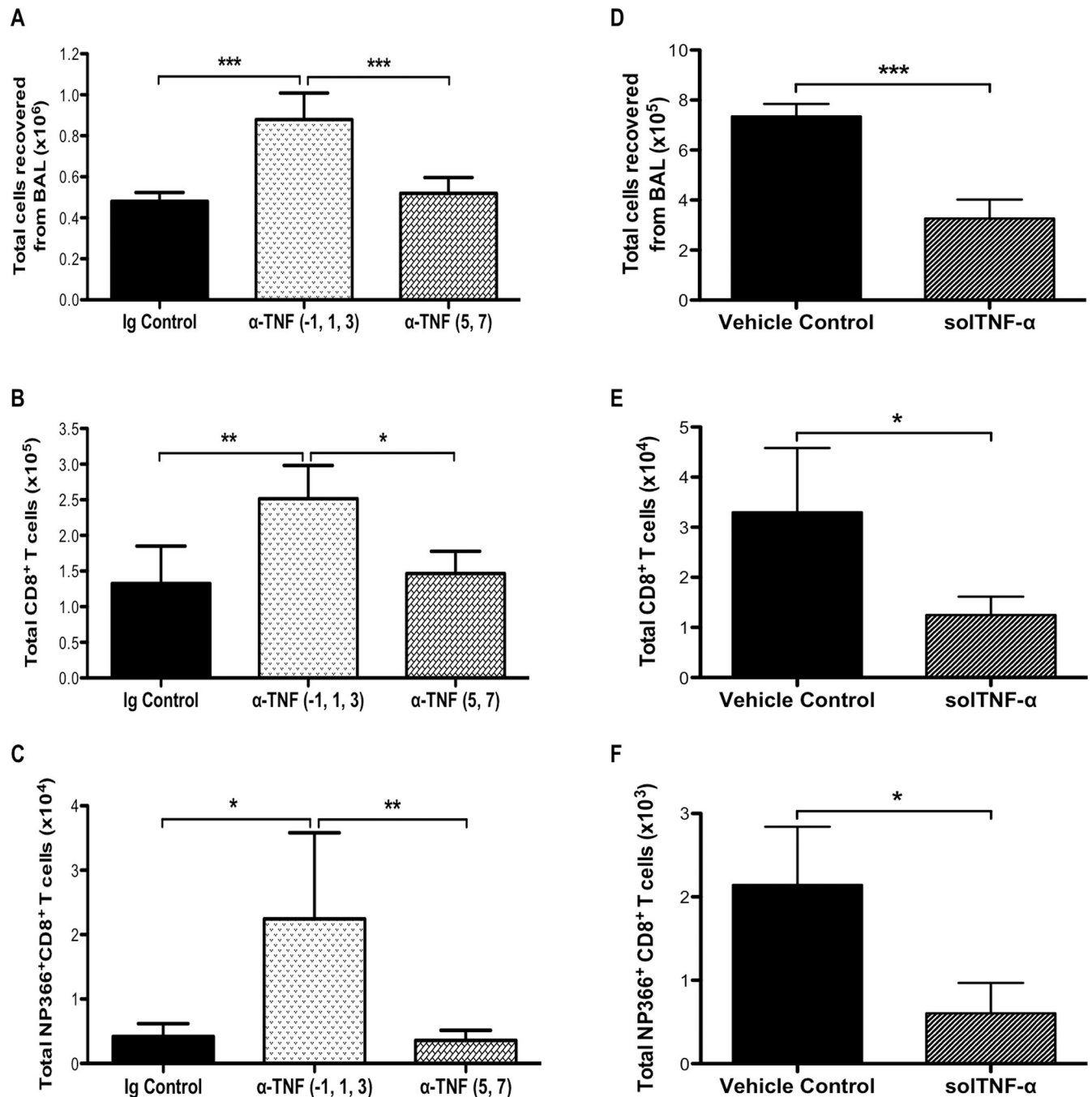


Figure 8. TNF- α expression is required early during infection to limit the size of the T-cell response

WT mice received anti-TNF- α or IgG1 control by intraperitoneal injection on days -1, 1, and 3 or days 5 and 7 post-infection. (A) Total number of viable cells recovered from the airways 8 days post-infection. Flow cytometry was used to analyze the total number of (B) CD8⁺ and (C) NP₃₆₆₋₃₇₄-specific CD8⁺ T cells. (D) Alternatively, memTNF^{1-9, K11E} KI mice were intranasally administered 2 μ g mouse recombinant solTNF- α at the time of infection and the total number of viable cells from the airways was enumerated on day 8 post-infection. Flow cytometry was used to analyze the total number of (E) CD8⁺ and (F)

NP₃₆₆₋₃₇₄-specific CD8⁺ T cells. Data represents mean \pm standard deviation. Each group consists of 3–5 mice per group. Data are representative of two independent experiments. * P <0.05, ** P <0.01, *** P <0.005.